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*A triple entente?*

# Interplay between alphaherpesviruses and interferon in sensory neuronal cells

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***About the cover***

The cover shows the interaction between an alphaherpesvirus and a neuron with the interferon caught in between. The electron microscopic picture of the virus was provided by T. Mettenleiter

The cover was designed by Alexander Ketele, the father of a very close friend of mine, Naomi Ketele. I am very thankful for the energy that he put in to make this design. Thank you very much!

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## List of abbreviations

| | |
|---|---|
| ActD | actinomycin D |
| BoHV-1 | bovine herpes virus 1 |
| CHX | cycloheximide |
| CoREST | Corepressor of REST |
| DRG | dorsal root ganglion |
| E genes | early genes |
| eIF2α | eukaryotic translation initiation factor 2 |
| GAS | gamma-activation sequence |
| HAT | histone acetyl transferase |
| HD | histone demethylase |
| HDAC | histone deacetylase |
| HMT | histone methyl transferase |
| hpi | hours post infection |
| HSV-1 | Herpes Simplex virus Type I |
| HSV-2 | Herpes Simplex virus Type 2 |
| IE genes | immediate-early genes |
| IFN | interferon |
| IRF | IFN regulatory factor |
| ISG | interferon-stimulated genes |
| ISRE | IFN-stimulated response element |
| LAT | latency-associated transcript |
| L genes | late genes |
| miRNA | microRNA |
| MOI | multiplicity of infection |
| ND10 | nuclear domain 10 |
| OAS | 2’5’-oligoadenylate synthetase |
| PAA | phosphonoacetic acid |

| | |
|---|---|
| pDC | plasmacytoid dendritic cells |
| PFU | plaque forming unit |
| PKR | protein kinase R |
| PML | Promyelocytic leukaemia |
| PNS | peripheral nervous system |
| PP1 | protein phosphatase 1 |
| PRV | Pseudorabies virus |
| REST | Repressor Element-1 Silencing Transcription factor |
| RT-PCR | reverse transcription polymerase chain reaction |
| RT-qPCR | quantitative reverse transcription polymerase chain reaction |
| ST | swine testicle |
| TG | trigeminal ganglion |
| TGN | *trans*-Golgi network |
| TIR | Toll-interleukin-1 receptor |
| TLR | Toll-like receptor |
| TSA | trichostatin A |
| VZV | Varicella Zoster virus |
| WT | wild type |

## Chapter 1: Introduction

## 1.1 Herpes Simplex virus type I and Pseudorabies virus

### 1.1.1 Introduction

The human herpes simplex virus type I (HSV-1) and the porcine pseudorabies virus (PRV), also known as Aujeszky's disease virus or suid herpes virus type I (SHV-1) both belong to the family of the *Herpesviridae*. The members of the *Herpesviridae* all share biological characteristics, although there are important differences between the subfamilies. All herpesviruses have a double-stranded DNA genome, a virion size of 200 – 250 nm and a structure that consists of a capsid, tegument and envelope. They all encode their own enzymatic machinery for replication and DNA synthesis, and capsid formation and encapsidation of viral genomes take place in the nucleus. They also all undergo a latent phase in their lifecycle (Roizman and Pellet, 2001).

Based on their biological properties, genome content and organization, herpesviruses are mainly subdivided in three major subfamilies: *Alphaherpesvirinae, Betaherpesvirinae* and *Gammaherpesvirinae*. The subfamilies differ in cell type where they establish latency and the duration of their replication cycle. The alphaherpesviruses, to which HSV-1 and PRV belong, have the broadest host range, replicate rapidly and establish latency mainly in sensory neurons. Betaherpesviruses have the most restricted host range, replicate very slowly and establish latency in a number of tissues including secretory glands, kidneys and lymphoreticular cells. Gammaherpesviruses have a restricted host range and establish latency in lymphoid tissue (Roizman and Pellet, 2001).

There are three important human alphaherpesviruses: herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2) and varicella zoster virus (VZV). Other important animal alphaherpesviruses besides the porcine PRV are equine herpesvirus 1 and 4 (EHV-1 and EHV-4), bovine herpesvirus 1 and 5 (BoHV-1 and BoHV-5), avian herpesvirus Marek's disease virus (MDV), canine herpesvirus 1 (CHV-1) and feline herpesvirus 1 (FPV-1) (Roizman and Pellet, 2001).

### 1.1.2 Pathogenesis

Herpes simplex has two virus variants: HSV-1 and HSV-2. The most common symptoms associated with HSV-1 are recurrent cold sores, but the virus may also cause recurrent ocular infections that represent the leading infectious cause of corneal blindness in the industrialized world. HSV-1 rarely causes encephalitis, which can be fatal. A large part of the human population is seropositive for HSV-1 (Jones, 2003). HSV-2 is the major cause of

recurrent genital lesions, although these may also be caused by HSV-1, but often causes asymptomatic infections.

PRV is the causative agent of Aujeszky's disease in pigs and is highly pathogenic for mammals except higher primates and humans. Pigs are the only susceptible animals that survive a PRV infection and are therefore considered as the natural host (Gustafson, 1986; Pensaert and Kluge, 1989). The severity of the disease depends on the age of the pig and the immunological status. Neonatal piglets suffer from nervous system disorders (which may be fatal) while older pigs show respiratory and reproductive problems (Baskerville, 1973; Akkermans, 1976).

The primary site of infection is located at the upper respiratory tract from where the virus spreads via blood, lymph and nerves to the central nervous system and internal organs, which represent the secondary sites of infection (Sabo et al., 1969; Baskerville, 1973; Miry and Pensaert, 1989).

### 1.1.3 Virion structure

All herpesviruses share the same virion structure and have a similar virion size of 200 to 250nm. The virion consists of four structural elements (Fig. 1). The genome consists of a double-stranded lineair DNA molecule of approximately 144kbp for PRV and 152kbp for HSV-1 (Ben-Porat and Kaplan, 1985; Roizman et al., 1992). The PRV genome sequence consists of a unique long segment ($U_L$) and a unique short segment ($U_S$) which is flanked by two inverted repeats, the terminal repeat sequence (TRS) and the internal repeat sequence (IRS) (Fig. 1C). The HSV genome has two repeat regions on both termini and a repeat region that is inserted in an inverted orientation separating the $U_L$ and $U_S$ regions (Fig. 1D). The genes are designated as UL or US, dependent on the location of the gene within the genome, followed by a number that indicates the place of the gene in the genome (Ben-Porat and Kaplan, 1985). The HSV-1 genome contains 80 open reading frames (ORFs) while PRV only encodes 72 ORFs. The genome sequence of alphaherpesviruses is highly conserved, implying that most viruses have orthologous proteins. In fact, PRV does not encode a single unique gene that is not present in other herpesviruses (Klupp et al., 2004; Pomeranz et al., 2005).

The genome is surrounded by an icosahedral capsid that consists of 162 capsomers. The capsid together with the genome is called the nucleocapsid (Mettenleiter, 2000). The major capsid protein VP5 is encoded by UL19 which is conserved among herpesviruses (Newcomb et al., 1999). The nucleocapsid is surrounded by an amorphous electron dense protein layer, the tegument. The inner layer of the tegument is tightly associated with capsid proteins and the outer layer is more asymmetrical and heterogeneous and is located beneath the envelope (Grunewald et al., 2003). The envelope forms the outer layer of the virion and consists of a double phospholipid layer. This layer originates from budding of the capsids in vesicles derived from the *trans*-Golgi network (Mettenleiter, 2006; Mettenleiter and Minson, 2006).

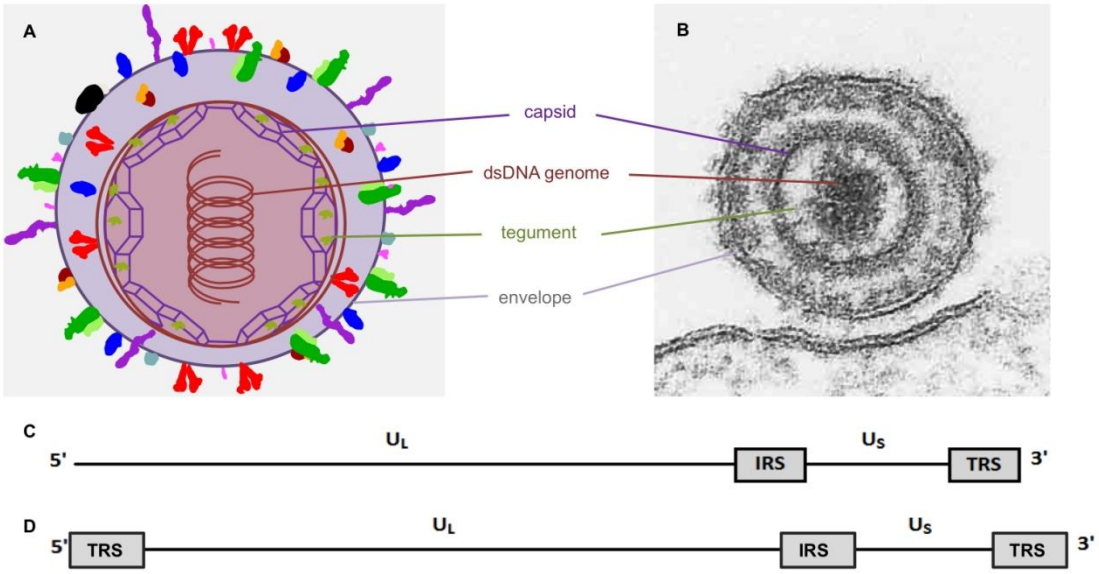


**Figure 1: A. Schematic representation of an alphaherpesvirus virion (adapted from Nauwynck et al., 2007). B. Transmission electron photomicrograph of a pseudorabies virion (Granzow et al., 1997). C. Schematic representation of the PRV genome structure. D. Schematic representation of the HSV genome structure. $U_L$ = unique long segment; $U_S$ = unique short segment; IRS = internal repeat sequence; TRS = terminal repeat sequence.**

### 1.1.4 Replication cycle

The alphaherpesvirus replication cycle is composed of several steps: virus entry, transport of the capsid to the nucleus, transcription and DNA replication, virion assembly and finally egress. Figure 2 shows a schematic representation supported with electron micrographs of all the respective stages of the infection cycle of PRV. Details on the replication cycles are based on data obtained from both HSV-1 and PRV.

*Entry (Fig. 2: step 1-2)*

Virion attachment to the plasma membrane occurs in two stages. First, an unstable binding is established between the cellular heparan sulphate and the viral glycoprotein gC and, to a lesser exent, gB (Mettenleiter et al., 1990). This is followed by a stable interaction between gD and its cellular receptor. Although the labile binding significantly enhances viral infection, it is in fact not required for infection (Mettenleiter et al., 1990; Karger et al., 1995; Immergluck et al., 1998; Shukla and Spear, 2001; Spear and Longnecker, 2003). To date, the known binding partners for gD during entry are divided in three categories: members of the TNF receptor family, members of the immunoglobulin superfamily and specific sites in heparan sulphate moieties created by 3-O-sulfotransferases (Geraghty et al., 1998; Shukla et al., 1999; Spear et al., 2000; Heldwein and Krummenacher, 2008). For PRV, in the immunoglobulin superfamily, three cellular receptors have been identified: nectin-1, nectin-2, and poliovirus receptor CD155 (Spear et al., 2000). CD155 is not used by HSV for entry while nectin-1 and nectin-2 are. Other HSV gD receptors include the TNF receptor family member (HVEM) and heparan sulphate modified by 3-O-sulfotransferase (Nixdorf et al., 1999; Campadelli-Fiume et al., 2000; Spear et al., 2000; Mettenleiter, 2002a; Spear and Longnecker, 2003).

More recently, it was described that besides gD also gB appears to interact with one or more receptors during entry of the virus. The paired immunoglobulin-like type 2 receptor PILRα acts as a coreceptor that associates with gB (Satoh et al., 2008). In addition, gB has also been reported to interact with lipid membranes (Hannah et al., 2009). Recently, it was described that gB can also interact with the non-muscle myosine heavy chain IIA (NMHC-IIA) to mediate entry in naturally permissive cells (Arii et al., 2010).

In order to mediate fusion between the envelope and the cellular membrane after attachment, several glycoproteins are required: gB, gD and gH-gL (Rauh and Mettenleiter, 1991; Mettenleiter and Spear, 1994; Klupp et al., 1997). The gB and gH-gL glycoproteins are essential for fusion, as mutants lacking these proteins are not able to enter the cell (Rauh and Mettenleiter, 1991; Rauh et al., 1991; Peeters et al., 1992a; Klupp et al., 1997; Turner et al., 1998; Mata et al., 2001; Gianni et al., 2006; Reske et al., 2007; Subramanian and Geraghty, 2007). Although direct fusion between plasma membrane and viral envelope is still considered as the major route of entry, more recent research points towards endocytosis as a possible way of viral entrance in several cell types (Nicola et al., 2003; Gianni et al., 2004; Nicola and Straus, 2004; Milne et al., 2005; Nicola et al., 2005; Clement et al., 2006; Frampton et al., 2007; Hambleton et al., 2007).

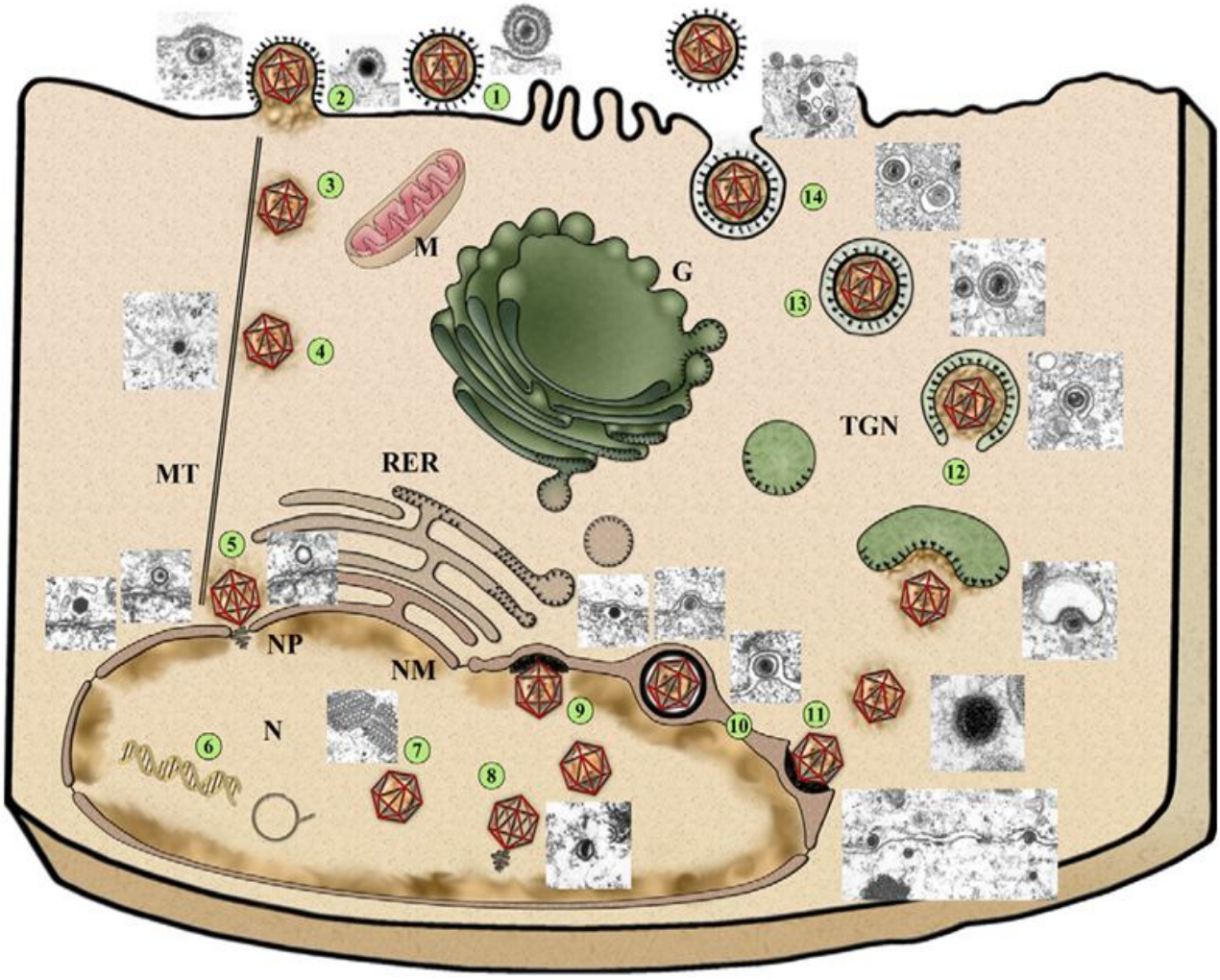


**Figure 2: Alphaherpesvirus replication cycle. Virions attach (1) and penetrate (2) the plasma membrane releasing the nucleocapsid surrounded by tegument into the cytoplasma. Capsids are then transported to the nucleus (N) (3) via interaction with microtubules (MT) (4), and dock at the nuclear pore (NP) (5) where the viral genome is released into the nucleus. Here, transcription of viral genes and genome replication occur (6) whereafter newly formed viral genomes are packaged during encapsidation (8) in newly formed capsids (7). These leave the nucleus by budding at the inner nuclear membrane (INM) (9), followed by fusion of the envelope of these primary virions that are located in the perinuclear space (10) with the outer nuclear membrane (ONM) (11). Secondary envelopment occurs through budding into vesicles of the *trans*-Golgi network (TGN) (12) containing viral glycoproteins, resulting in an enveloped virion. After transport to the plasma membrane (13), vesicle and plasma membrane fuse, releasing a mature, enveloped virion from the cell (14). (adapted from Mettenleiter et al., 2009).**

*Transport to the nucleus (Fig. 2: steps 3-5)*

Upon entry in the cytoplasm, capsids are rapidly transported to the nuclear pore complexes, where they release the viral DNA genome into the nucleus. The transport through the cytosol is mediated via microtubule-based transport. Capsids engage dynein, a motor protein directed to the minus-end of the microtubules, to efficiently migrate from the periphery to the nucleus (Sodeik et al., 1997; Smith and Enquist, 2002). For HSV-1, the proteins VP11/12, VP26, UL9 and/or UL34 are proposed to recruit dynein to capsids as these proteins all have been shown to be able to interact with dynein (Purves et al., 1992; Ye et al., 2000; Martinez-Moreno et al., 2003; Douglas et al., 2004; Diefenbach et al., 2008). However, UL34 is absent in mature PRV virions and is not essential during PRV- and HSV-1 infection (Klupp et al., 2000; Roller et al., 2000; Fuchs et al., 2002b). On the other hand, mutants lacking VP26 are still transported to the nucleus in a dynein-dependent manner, indicating that UL34 and/or VP26 are less likely candidates to mediate alphaherpesviruses transport along microtubules (Antinone et al., 2006; Dohner et al., 2006).

When the virions reach the nucleus, a docking complex is formed mediated by importin β and the VP1/2 tegument protein (Ojala et al., 2000). Thereafter, viral DNA is rapidly released into the nucleus (Batterson and Roizman, 1983; Sodeik et al., 1997; Ojala et al., 2000).

*Gene expression (Fig.2: step 6)*

Upon arrival in the nucleus, the viral genome circularizes and is transcribed in a tightly regulated cascade manner (Ben-Porat and Kaplan, 1985). Viral replication proceeds through the rolling circle mechanism. Viral genes can be divided into three categories: immediate-early (IE), early (E) and late (L) genes. The latter can sometimes be subdivided into early-late and true-late. Expression of immediate-early and early genes occurs before onset of viral DNA replication and the expression of early-late genes starts before DNA replication but reaches its peak of transcription during replication. The expression of true-late genes only starts after replication has initiated.

First, IE genes are transcribed very rapidly upon delivery of the genome into the nucleus. These genes encode transcriptional regulatory proteins and therefore are necessary to activate the transcription cascade of E and L genes. The transcription of IE genes is initiated by the host cell machinary together with the viral protein VP16 (Pomeranz et al., 2005). PRV only encodes one IE gene, IE180, while HSV-1 encodes 5 IE genes: ICP0, ICP4 (the orthologue of IE180), ICP22, ICP27 and ICP47 (Cheung, 1989; Roizman and Knipe, 2001). IE180 promotes expression of US4 (gG), UL12 (alkaline nuclease), UL22 (gH), UL23

(thymidine kinase) and UL41 (viral host shut off). The E gene products are mainly involved in nucleotide metabolism (UL23, UL39/40, UL50) and DNA synthesis (UL5, UL8, UL9, UL29, UL30, UL42, UL52) (Kit et al., 1987; Kaliman et al., 1994; Berthomme et al., 1995; Jons et al., 1997; Lehman and Boehmer, 1999). Finally, expression of late genes is triggered by DNA replication. These late genes mainly encode structural proteins involved in assembly and egress (Mettenleiter, 2000; Roizman and Knipe, 2001; Pomeranz et al., 2005).

*Assembly and egress (Fig. 2: steps 7-14)*

Upon replication, capsid constituents are imported into the nucleus to build the new capsids around two scaffolding proteins (UL26 and UL26.5). During DNA encapsidation, the long linear concatemeric viral genome is cleaved into monomeric units and is simultaneously packaged into newly formed capsids by pulling them through a cylindrical entry pore encoded by UL6 (Ladin et al., 1980; Ben-Porat et al., 1984; Kwong and Frenkel, 1989; White et al., 2003; Cardone et al., 2007).

Herpesviruses are too large to pass through the nuclear pore. In order to cross the nuclear envelope, herpesviruses utilize a unique envelopment/de-envelopment process at the inner and outer nuclear membrane, respectively (Hofemeister and O'hare, 2008). First, the nucleocapsids acquire their primary envelope and tegument by budding through the inner nuclear membrane (INM) leaving them in the perinuclear space. The viral proteins UL31 and UL34 are required for primary envelopment, and are found in all three herpesvirus subfamilies indicating that the process of primary envelopment is highly conserved (Ye and Roizman, 2000; Fuchs et al., 2002b; Mettenleiter, 2006). Expression of UL31/UL34 alters the nuclear lamina architecture allowing the viral capsids to enter the site of envelopment (Reynolds et al., 2004; Simpson-Holley et al., 2004), and expression of the UL31 and UL34 proteins of PRV, without other viral proteins, is sufficient to generate an envelopment-like process at the inner nuclear membrane (Klupp et al., 2007). Primary enveloped virus particles that are located in the perinuclear space will then undergo a fusion event with the outer nuclear membrane (ONM), releasing the de-enveloped capsid in the cytosol. The viral protein US3 plays a poorly understood role during de-envelopment (Klupp et al., 2001; Reynolds et al., 2002). This de-envelopment process appears to be very rapid as very few enveloped capsids are observed in the perinuclear space (Skepper et al., 2001; Mettenleiter, 2002b). In HSV-1, but not in PRV, a defect in nuclear egress was also observed in the absence of gB and gH (Farnsworth et al., 2007). Although nucleocapsids that reach the cytoplasm appear ‘naked’, they carry tegument proteins US3, UL36 and UL37 (Fuchs et al., 2002a). Upon reaching the cytoplasm, tegumentation and final envelopment at vesicles of the *trans*-Golgi network (TGN) occur (Mettenleiter, 2004). UL36 is the major component of

the inner tegument and interacts with the minor capsid component UL25 (Coller et al., 2007). The UL11 tegument protein is thought to influence secondary envelopment by directing tegument proteins and capsids to the TGN budding site. The glycoprotein gM is involved in accumulation of the unusually diverse set of alphaherpesvirus envelope glycoproteins to the TGN budding site (Kopp et al., 2003; Kopp et al., 2004; Leege et al., 2009). Finally, mature enveloped virions are released from the host cell by exocytosis. In PRV, the viral proteins UL20 and gK seem to be involved in this process (Fuchs et al., 1997; Klupp et al., 1998). However, in HSV-1, data are contradictory as some groups report the same function for UL20 and gK as in PRV, while other groups argue that these proteins function during secondary envelopment (Baines et al., 1991; Avitabile et al., 1994; Foster et al., 2004a; Foster et al., 2004b; Fulmer et al., 2007).

*Intercellular spread*

Alphaherpesvirus spread from an infected to an uninfected cell can occur through two main routes. Release of progeny virions from an infected cell into the extracellular space followed by attachment and entry in an uninfected neighbouring cell is the traditional way of viral spread. Additionally, alphaherpesviruses may also spread in a cell-associated way, which may be more efficient and rapid, and shields them from different antiviral components in the extracellular space, including virus-neutralizing antibodies. Several ways of cell-to-cell spread have been described. When an infected cell and an uninfected cell are in direct contact, spread can occur through fusion of plasma membranes of both cells, resulting in syncytia formation. This process is mediated by glycoproteins on the cell surface in a process that resembles the fusion between the viral envelope and the plasma membrane during viral entry. It is intriguing that in PRV, but not in HSV-1, gD is not required for cell-to-cell spread while it is essential for viral entry (Rauh and Mettenleiter, 1991; Peeters et al., 1992b; Ch'ng et al., 2007). In neurons, PRV gD induces varicosities (synaptic boutons) during viral infection. These varicosities have been described to serve as sites for viral cell-to-cell spread (De Regge et al., 2006). The glycoprotein gE is involved in cell-to-cell spread in polarized epithelial cells by sorting virions to the cell junctions (Zsak et al., 1992; Johnson et al., 2001). The US3 protein of PRV and different other alphaherpesviruses induces protrusions that connect distant cells to infected cells, allowing efficient intercellular viral spread (Favoreel et al., 2005).

## 1.2 Alphaherpesvirus interaction with sensory neuronal cells

### 1.2.1 Sensory neurons

The peripheral nervous system (PNS) consists of nerves and ganglia outside the brain and spinal cord. The main function of the PNS is to connect the central nervous system (CNS) to the limbs and organs. Sensory neurons that make part of the PNS are afferent neurons that transmit nerve impulses towards the CNS. Figure 3.A. shows the schematic representation of a sensory neuron. Sensory neurons are of special interest in the context of alphaherpesvirus biology as they represent the predominant site for the establishment of latency for most of these viruses. Several alphaherpesviruses have a tropism for trigeminal ganglion neurons (e.g. HSV-1, PRV and BoHV-1), dorsal root ganglion neurons (e.g. VZV) or sacral ganglion neurons (e.g. HSV-2) (Gutekunst et al., 1980; Ackermann et al., 1982; Croen et al., 1987). Localisation of the dorsal root ganglia, sacral ganglia, and trigeminal ganglia can be seen in Figure 3 and 4.

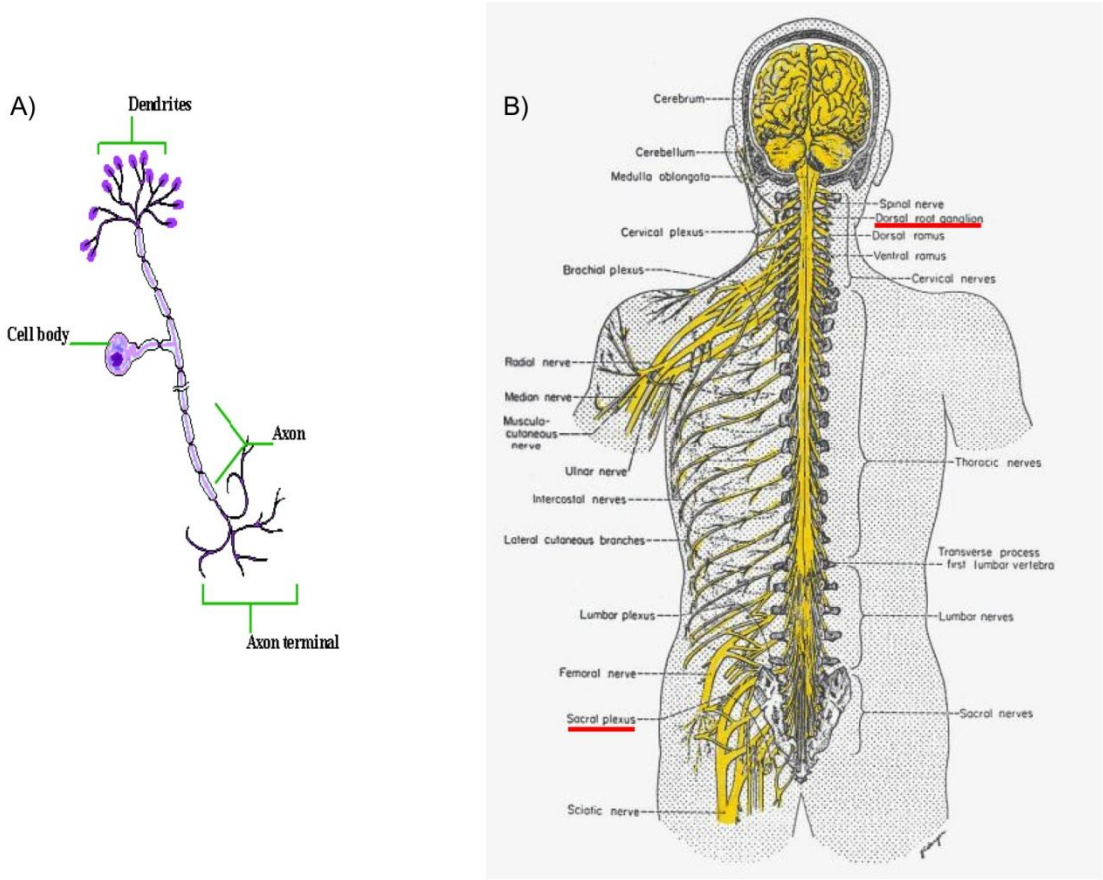


**Figure 3: A) Schematic representation of a sensory neuron and B) representation of the most important human nerves on the dorsal side of the body. Red underlined: dorsal root ganglia and sacral ganglia.**

*Trigeminal ganglion*

The name trigeminal is derived from ‘tri’ and ‘geminus’ which means three twins and originates from the fact that three major nerve branches originate from the trigeminal ganglion: the ophthalmic nerve, the maxillary nerve and the mandibular nerve. The ophthalmic and maxillary nerves are exclusively sensory, while the mandibular nerve contains both sensory and motor neurons. The three branches converge in the trigeminal ganglion which contains the cell bodies of incoming sensory nerve fibers. The trigeminal ganglion (TG) is analogous to the dorsal root ganglia of the spinal cord, which contains the cell bodies of incoming sensory fibers from the rest of the body. TG neurons are pseudo-unipolar with one part of the neuronal axon directed to the CNS and the other part directed to the periphery (Firbas et al., 1995; Marieb et al., 2005). Figure 4 shows the localization of the three nerve branches that end up in the trigeminal ganglion and the region of the face innervated by the different nerve branches. The ophthalmic nerve conveys sensory impulses from the skin of the anterior scalp, upper eyelid, the conjunctiva and the cornea of the eye, the nose and the nasal mucosa and lacrimal gland. The maxillary nerve carries information from the lower eyelid, the palate, the nasal mucosa, the upper lip and the cheek. The mandibular nerve transports sensory information from the anterior tongue, skin of chin, lower lip and temporal region of the scalp. The motor functions regulated by the mandibular nerve comprise biting, chewing and swallowing (Marieb et al., 2005).

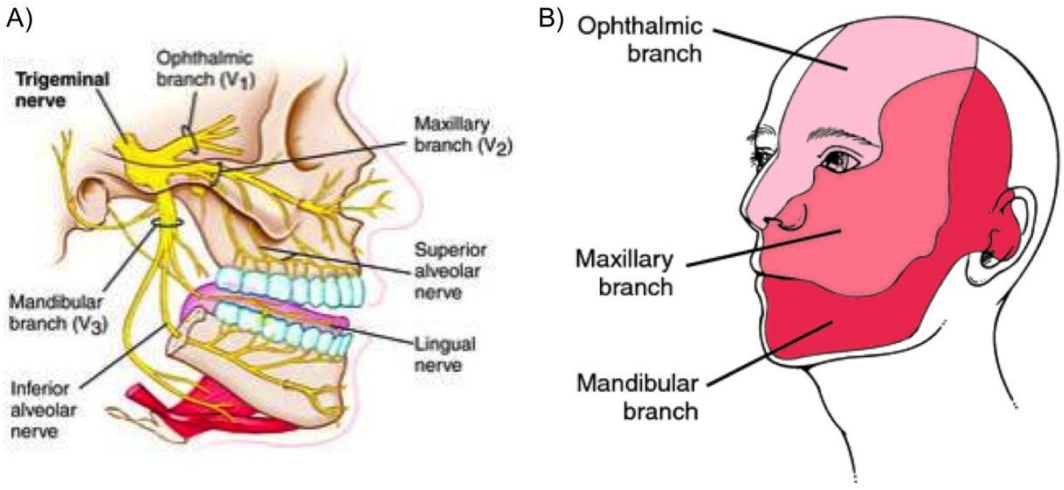


**Figure 4: A) schematic representation of the trigeminal nerves and B) the region they innervate.**

*Alphaherpesvirus infection of sensory neurons*

HSV-1 and PRV share the ability to establish latency in neurons of the trigeminal ganglion. On average, cell bodies (containing the cell nucleus), are approximately 10cm away from the axon termini that innervate the face (Fig.4). HSV-1 and PRV make use of microtubule-based retrograde axonal transport to overcome this huge distance to deliver their genome in the nucleus (Sodeik et al., 1997; Diefenbach et al., 2008). After reaching the nucleus, the viruses can either start a lytic infection which results in the formation of newly formed virus particles or enter latency which consists of a dormant state of infection without the production of infectious virus (Roizman and Knipe, 2001). The general replication cycle of alphaherpesviruses has been discussed in 1.1.4, so only the major differences between typicial replication in e.g. epithelial cells and infection of neurons will be addressed here.

As the distance between the plasma membrane and the nucleus is much larger than in epithelial cells, the transport along the axons results in the inefficient ability of different viral tegument components, e.g. VP16, to reach the nucleus. Since VP16 is a viral transactivator, this neuron-specific feature is thought to be of special interest for the ability of the neurons to be prone to latent alphaherpesvirus infections, which will be discussed in the next part of the introduction (1.2.2).

The biggest difference between infection of epithelial cells and neurons lies in the mode of viral egress from an infected cell and spread to other cells. During a typical replication cycle, as in epithelial cells, progeny capsids that are produced in the nucleus and released in the cytoplasm, are thought to acquire their envelope by budding into vesicles derived from the *trans*-Golgi network, followed by release of the virions via exocytosis. In neurons, progeny virus particles need to travel back from the nucleus to the periphery along the axon, using anterograde microtubule-based transport. However, there is ongoing debate on the composition of virus particles travelling down the axon. Two models have been proposed regarding the transport in anterograde direction: the traditional model and the subvirion model. In the traditional (married) model, progeny viruses become fully assembled in the neuronal cell body by budding of the capsids in the *trans*-Golgi followed by anterograde transport of the enveloped virions carried in vesicles, towards the axon termini (Kristensson et al., 1974; Card et al., 1993; Tomishima and Enquist, 2001) (Figure 5). This model corresponds to the mode of egress in non-neuronal cells (1.1.4). Data to support this model come from the electron microscopic detection of fully enveloped PRV and HSV-1 virions in vesicles in axons of different kinds of neurons: rat superior cervical ganglion neurons (SCG) and human and rat dorsal root ganglion neurons (DRG) (Marchand and

Schwab, 1986; Lycke et al., 1988; Card et al., 1993; Ch'ng and Enquist, 2005; Del Rio et al., 2005).

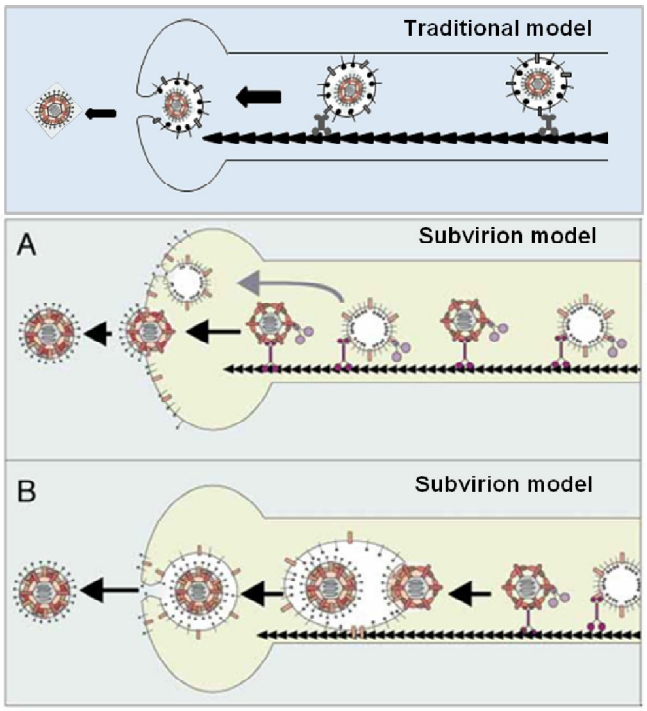


**Figure 5: Schematic representation of alphaherpesvirus entry and egress at axon termini.** The traditional model of egress shows anterograde transport of fully enveloped virions along the axon. The subvirion model comprises two proposals: A) capsids and tegument are transported separate from glycoprotein-containing vesicles that fuse with the plasma membrane prior to budding of the capsid to obtain its secondary envelope. B) capsids acquire their secondary envelope through budding in an glycoprotein-containing endosome present in the axon terminus and are subsequently released through exocytosis. (Adapted from Tomishima et al., 2001)

Another argument pro the traditional model is the fact that infectious PRV could be isolated from mid-axons of porcine TG neurons (Kritas et al., 1994b; Kritas et al., 1994a; Kritas et al., 1995). As it is irrefutable that incoming virions loose their envelope during entry, the former data cannot be explained by visualisation or detection of incoming virus during retrograde transport (Lycke et al., 1984; Lycke et al., 1988; Card et al., 1993; Tomishima and Enquist, 2002). More recent studies using dual-fluorescent PRV viruses (carrying a fluorescently tagged capsid and envelope) also support the traditional model (Del Rio et al., 2005; Luxton et al., 2005; Antinone et al., 2006). The alternative model, the subvirion (separate) model, was proposed for the first time in 1994 by the Cunnigham lab (Penfold et al., 1994). This model proposes that the envelope proteins are transported to the axon termini in glycoprotein-containing vesicles separate from capsids which do not use vesicles for their transport. The initial data supporting this hypothesis came from an electron microscopy study that detected HSV-1 particles, at a time point when a majority of particles would be considered to be engaged in anterograde transport, as unenveloped capsids in

axons of human fetal DRG neurons (Penfold et al., 1994). Other studies using human or rat DRGs showed that unenveloped capsids surrounded by tegument proteins were located adjacent to microtubules, whereas glycoproteins and other tegument proteins were present in vesicles without capsids (Holland et al., 1999; Miranda-Saksena et al., 2000). More recent studies showed both for PRV and HSV-1 that capsids were separated from vesicles containing glycoproteins (Miranda-Saksena et al., 2000; Smith et al., 2001; Enquist et al., 2002; Potel et al., 2003; Snyder et al., 2006; Snyder et al., 2007). Two models are proposed as part of the subvirion model (Fig. 5. A and B). The first hypothesis states that vesicles containing envelope proteins fuse with the axolemma, followed by budding of the capsid through this plasma membrane to release infectious virus. The second hypothesis states that vesicles containing glycoproteins fuse with endosomes present in the axon terminus, followed by budding of capsids in this endosome releasing infectious virus by exocytosis (Tomishima and Enquist, 2001).

It is still not clear which model of anterograde transport is correct, although it is possible that both models coexist. One possible explanation for the observed differences lies in the experimental setup. Different viruses, different neurons and different species make it extremely hard to evaluate the data in comparison to each other. Also, analyses on fixed samples (e.g. electron microscopy and confocal microscopy) should be interpreted with care as (i) they may contain fixation artefacts and (ii) they are more difficult to interpret since it is complicated to distinguish in fixed samples whether a particle is engaging in anterograde transport, retrograde transport or is not moving at all. A recently published study showed that anterograde transport of complete virions occurred for HSV-1, HSV-2 and PRV in rat SCGs. Fully assembled virus particles were detected intracellularly within vesicles in proximal and mid-axons adjacent to microtubules (Huang et al., 2011). Also, a recent report showed that the HSV-1 strain KOS, often used in studies addressing neuronal transport, carries a defective US9 gene (Negatsch et al., 2011). As US9 is essential for virus anterograde transport (Feierbach et al., 2007), this is likely to have affected results and conclusions concerning HSV-1 anterograde transport.

Alphaherpesviruses are transported via the axons back to the periphery where the virus can spread to epithelial cells and hence cause recurrent symptoms. Different studies have shown that the virus not only leaves the axon at the axon terminus, but also may exit the axon at pre-synaptic boutons (varicosities) along the axon shaft (Ch'ng and Enquist, 2005; De Regge et al., 2006; Saksena et al., 2006). For PRV, it has been shown that the viral glycoprotein gD induces the formation of these varicosities through Cdc42 and p38 MAPK signalling pathways (De Regge et al., 2006).

### 1.2.2 Latency/ reactivation

Latency is a central aspect of the life cycle of herpesviruses. Neurons are the most predominant site of latency for most alphaherpesviruses (see 1.2.1). Latency in this particular cell type is a strategy used by the virus to survive in the host for its entire lifetime and enables the virus to escape the host immune system. Latency is characterized by the absence of the production of infectious virus particles while the viral genome is present in the cell and can be reactivated upon specific stimuli which may give rise to recurrent viral spread and symptoms (e.g. cold sores and genital lesions by HSV). For some alphaherpesviruses (e.g. HSV and PRV but not VZV), another feature of latency is the presence of specific latency-associated transcripts (LATs) but the absence of any detectable viral protein. The exact importance of LATs for latency is not yet resolved. LATs have been attributed an anti-apoptotic function through a microRNA (miRNA) encoded by the first exon of the LAT region (Gupta et al., 2006). Furthermore miRNAs were found directed against the immediate-early genes ICP0 and ICP4, indicative for the involvement of LATs in suppressing lytic gene expression, and thereby promoting latency. Other miRNAs in the LAT region are directed against ICP34.5, an important neurovirulence factor (Umbach et al., 2008). The latency/reactivation cycle can be subdivided in three phases: (i) the establishment of latency during which a dormant state is evoked followed by (ii) the maintenance of latency during which the virus is kept dormant and, upon specific stimuli, (iii) reactivation which may lead to the production of new virus particles. It has become increasingly clear that latency of alphaherpesviruses is dependent on the interplay between neuron, virus and immune system. The following sections will discuss this intriguing triple entente.

*Interaction between neuron, immune system and alphaherpesviruses*

Several data indicate that alphaherpesvirus latency in neurons is the result of a delicate balance between the neurons, the immune system and the virus itself. It has been put forward that neurons represent a repressive environment for alphaherpesvirus replication, thereby contributing substantially to latency establishment in this particular cell type. Some neuron-specific properties and immune components that have been proposed to contribute to latency will be discussed below.

Since in latently infected neurons the expression of IE genes is hampered, the hypothesis was put forward that a transcriptional block occurred in neuronal cells, preventing initiation of lytic infection (Hagmann et al., 1995). A central factor in the activation of lytic infection is the formation of a complex between the viral tegument protein VP16 and the cellular proteins Oct-1 and HCF-1, which will bind to the IE promoter sequence (Hagmann et

al., 1995). In neurons however, the formation of this essential complex is hampered. It was hypothesized that VP16 is largely lost during transport along the axons leading to a less efficient onset of productive infection (Roizman and Sears, 1987; Kristie and Roizman, 1988). However, a later study showed that latency was efficiently induced in the presence of a functional VP16 (Sears et al., 1991). It was also shown that HCF-1 is located predominantly in the cytoplasm in neurons whereas in non-neuronal cells it is present in the nucleus, where it can activate transcription (Kristie et al., 1999). In addition, the other critical cellular transcription factor, Oct-1, is only expressed in low abundance in neurons (Hagmann et al., 1995). Also very important in the context of latency is the fact that neurons are terminally differentiated and therefore express very few proteins required for DNA replication, which is necessary for viral replication (Nichol et al., 1996).

Besides the repressive environment of the neuron, the immune system also plays a central role in alphaherpesvirus latency in neurons. Indeed, for PRV, it has been shown that, *in vitro*, virtually all trigeminal ganglion neurons support initiation of productive infection (Geenen et al., 2005; Geenen et al., 2007). This indicates that the repressive properties of neurons are not the only factors in the equation to establish a latent infection. The interferon-inducible transcription repressing nuclear domains (ND10s) have been shown to restrict viral replication. However, several studies reported abnormal ND10 structures or the absence of ND10s in neurons (Hsu and Everett, 2001; Negorev et al., 2001).

Studies using mice have shown that HSV-1 antigens were detectable in TGs at 2dpi and that titers reached their peak around 3 to 5dpi, declining between day 7 and 10 where after antigens were no longer detectable (Shimeld et al., 1995; Liu et al., 1996; Kodukula et al., 1999). This pattern corroborates with the induction of the immune system. At 3dpi macrophages start infiltrating the TGs and at 5dpi γδ-T cells were very closely associated with neurons (Liu et al., 1996). A modest increase in $CD4^+$ cells and a large raise in $CD8^+$ cells were seen at 7dpi (Liu et al., 1996). From 3dpi onwards IFNγ and IL4 production appeared and from 7dpi IL10 and TNF production became evident (Liu et al., 1996), although different studies gave different results concerning the time course of cytokine production (Halford et al., 1997; Kodukula et al., 1999). It was also shown that high levels of IFNα in the TGs resulted in a strong reduction of viral titers at 3 and 6dpi (Carr et al., 1998). These combined studies indirectly suggest that infiltrating immune cells and cytokines of the innate immune system in the TG may be involved in establishment of latency. Since latency establishment is believed to occur within days post infection, it is less clear whether the adaptive immune system is also involved in the establishment phase of latency. However, the exact mechanism of establishment of latency still remains to be elucidated.

Several studies have reported that the presence of immune cells and cytokines in the TGs lasts longer than needed for the establishment of latency, and in fact appears to result in a sustained, low-level activation of the immune system in the ganglion. $CD4^+$ T cells, $CD8^+$ T cells and macrophages were found up to 92dpi in TGs of latently infected mice (Shimeld et al., 1995; Liu et al., 1996; Khanna et al., 2003). TNFα, amongst others, could also be detected until 92dpi (Liu et al., 1996). $CD8^+$ T cells are present in particularly high numbers and for a long time in the TGs, and are localized in close opposition to the infected neurons (Khanna et al., 2003). The importance of $CD8^+$ T cells most likely lies in their ability to repress reactivation, without killing the neurons. Indeed, $CD8^+$ T cells have been shown to inhibit reactivation of latent HSV-1 during explant of latently infected mouse TG (Liu et al., 2000). Also, mice deficient for $CD8^+$ T cells failed to control HSV-1 infection resulting in fatal encephalitis between 7 and 12dpi (Lang and Nikolich-Zugich, 2005). $CD8^+$ T cells release IFNγ as a potential non-cytolytic mode of suppressing virus replication (Guidotti and Chisari, 2001). Indeed, it was reported that administration of IFNγ to explanted latenly infected mouse TG was sufficient to prevent reactivation (Liu et al., 2001; Decman et al., 2005). The role of IFNγ in the maintenance of latency was confirmed in IFNγ knock-out mice, which showed a significantly higher rate of HSV reactivation (Minami et al., 2002). The constant presence of cytokines and immune cells point to the direction of a continuous attempt of the virus to reactivate and a subsequent rapid response of the immune system to prevent these initial reactivation events leading to production of infectious virus.

*Chromatin modifications*

(adapted from Van Opdenbosch N., Favoreel F.W. and Van de Walle G, 2011, Biology of the Cell, in press)

In herpesvirus biology, histone modifications are being studied with increasing interest, mainly because chromatin modifications appear to offer an epigenetic switch between lytic and latent infections (Minarovits, 2006; Knipe and Cliffe, 2008; Reeves, 2011). The most important histone modifications with regard to regulation of herpesvirus transcription are histone acetylations and methylations. Acetylation of histones results in open chromatin, which allows transcription, while deacetylation causes the chromatin to close and therefore block transcription. Tri-methylation can cause both activation and repression of transcription based on the position where this post-translational modification occurs. For example, tri-methylation of H3K9 and H3K27 is linked to transcriptional repression while tri-methylation of H3K4 and H3K36 causes transcriptional activation. Di-methylation of H3K4 and mono-methylation of H3K9 are also markers for transcriptional activation, while di-methylation of H3K9 again are linked to transcriptional repression

(Bedford and Clarke, 2009; Peserico and Simone, 2011). Clear differences have been observed in histone modifications on latent and lytic alphaherpesvirus genomes.

During latent HSV-1 infection, only a small, intron-containing part of the genome is available for transcription, leading to the expression of the latency-associated transcript (LAT). It was shown that the latent HSV-1 genome is highly associated with nucleosomes, including the LAT region, and it has been proposed that the histone composition may be a major regulator of HSV-1 latency (Deshmane and Fraser, 1989; Kubat et al., 2004). Indeed, ChIP assays of latent HSV-1 genomes revealed that the LAT region is associated with acetylated H3K9/K14, implying a transcriptionally active euchromatin structure, while the DNA polymerase region was not enriched with acetylated H3K9/K14, indicating a transcriptionally inactive chromatin structure. LAT by itself also affects the chromatine structure and therefore, affects latency. Early reports already indicated that LATs are able to repress lytic gene expression in sensory neurons (Garber et al., 1997). More recently, it was demonstrated that the LAT gene increases the levels of H3K9me2 and reduces the levels of H3K4me2 on lytic gene promoters, indicating that the LAT gene promotes a transcriptionally suppressed heterochromatin structure on lytic gene promoters (Wang et al., 2005). As mentioned higher, explanting mouse trigeminal ganglia containing latent HSV-1 results in virus reactivation (Stevens and Cook, 1971). Using this model, it was shown that the level of acetylated H3K9/K14 is reduced in the LAT enhancer at early times post explant, suggestive for silencing of the LAT region. Subsequently, the amount of LAT RNA diminishes and this is accompanied by an increased acetylation in the ICP0 promoter, which indicates the onset of reactivation (Amelio et al., 2006). Likewise, addition of the HDAC inhibitor sodium butyrate results in reduced acetylation of H3K9/K14 on both the LAT promoter and enhancer on the one hand and an increase in acetylation on the ICP0 and ICP4 promoters on the other hand, again pointing towards reactivation (Neumann et al., 2007). These findings are in line with previous data showing that addition of HDAC inhibitors led to the production of increased virus progeny in quiescently infected cells (Danaher et al., 2005). On latent HSV-1 genomes, both constitutive as well as facultative types of heterochromatin are present, as demonstrated by the presence of the facultative heterochromatin marker H3K27me3 and the constitutive marker H3K9me3. Interestingly, it was shown that in the absence of LAT, the latent genome undergoes a dramatic increase in H3K27me3, indicating that the viral genome contains more facultative heterochromatin, which likely renders lytic genes more prone to activation (Cliffe et al., 2009; Kwiatkowski et al., 2009).

Different immediate early and early viral proteins have been shown to interact with chromatin-remodeling complexes, thereby affecting gene transcription. The net result of

these interactions appears to be to promote lytic replication, and therefore perhaps reactivation from latency. The most notable alphaherpesvirus proteins involved in activating lytic gene expression through histone modifications are VP16, ICP0 and US3, which are discussed in more detail below.

VP16 is able to recruit the chromatin-remodeling and transcription activating complex SWI/SNF to the IE promoters (Memedula and Belmont, 2003; Herrera and Triezenberg, 2004). Importantly, VP16 has recently been suggested to be critically involved in reactivation of HSV-1 from latency in mice (Thompson et al., 2009). Due to the role of VP16 in recruiting chromatin-modifying proteins, the hypothesis arose that these coactivators are essential for IE gene expression during lytic infection and perhaps reactivation from latency. However, disrupting the expression of the SWI/SNF complex did not impair IE gene expression in HSV-1 infected cells, suggesting that these transcriptional coactivators are not required for the IE gene expression of nucleosome-associated HSV-1 genomes (Kutluay et al., 2009). As mentioned before, VP16 interacts with HCF-1 to assemble a transcription initiation complex. HCF-1 is also part of several chromatin modifying complexes including the Set1-MLL HMT complex, the LSD1 and mSin3-HDAC1/2HD complexes, and the PCAF HAT complex (Kolb and Kristie, 2008; Peng et al., 2010) and HCF-1 was shown to be essential for IE gene expression, both in HSV-1 and VZV (Narayanan et al., 2005). More specifically, VP16 recruits the HCF-1 cellular complex which includes hSet1 and MLL1 and this direct recruitment of hSet1-MLL to the IE promoters results in the loss of repressive (H3K9me3) and gain of activating (H3K4me3) chromatin methylation marks leading to activation of transcription (Huang et al., 2006; Narayanan et al., 2007). In addition, the histone demethylase (HD) LSD1 is also enrolled in the activating complex in an HCF-1 dependent manner (Liang et al., 2009). Recently, it was discovered that HCF-1 not only drives productive infection upon viral entry, but is also involved in HSV-1 reactivation from latency. Indeed, triggering of reactivation through the explant of latently infected mouse TG or scarification of the eyes of latently infected mice, resulted in rapid relocation of HCF-1 to the nucleus of sensory neurons where it participates in the onset of transcription (Kolb and Kristie, 2008; Whitlow and Kristie, 2009).

The immediate-early ICP0 protein is involved in the inhibition of histone deacetylases (HDACs) to prevent silencing of the viral genome and thus support lytic replication as the absence of ICP0 can partly be compensated for by the HDAC inhibitors trichostatin A and sodium butyrate (Hobbs and Deluca, 1999; Poon et al., 2002; Poon et al., 2003). In this way, ICP0 may promote lytic infection and reactivation from latency. During lytic replication, the VP16/HCF-1 complex will bind to the ICP0 promoter to initiate transcription of this gene and

other IE genes, while the early (E)- and late (L)- gene promoters are bound by the CoREST repressive complex which also includes HDAC1/2 and LSD1(Fig.6). Expression of ICP0 will then allow for transcription of the E- and L- genes via two ICP0 functions: (i) the destruction of ND10s by disruption of PML and (ii) dislocation of HDAC1/2 from the repressive CoREST/REST complex (Roizman, 2011) (Fig. 6). The current hypotheses on how these two functions of ICP0 are important for the regulation of gene activation/silencing will be discussed in more detail in the next paragraphs. In general it is stated that both functions of ICP0 are interdependent and largely depend on events in the ND10 (Gu and Roizman, 2009b).

First, ICP0 is involved in disruption of the ND10 (Everett and Maul, 1994). ND10s are considered as antiviral structures containing cellular proteins which suppress viral replication and which are upregulated by interferon (Tavalai and Stamminger, 2008). ICP0 is specifically involved in the proteasome-dependent degradation of proteins present in ND10s, namely ND10-associated proteins promyelocytic leukemia (PML), Sp100, ATRX and hDaxx (Chelbi-Alix and De Thé, 1999). Through its RING finger domain, ICP0 is able to bind to the ND10-associated cellular ubiquitin specific protease (USP) 7, which was originally named herpesvirus-associated ubiquitin specific protease (HAUSP) (Everett et al., 1997). ICP0 was shown to also directly interact with Bmal1, another protein that is localized in ND10s. Since Bmal1 associates with the transcriptional coactivator and histone acetyl transferase (HAT) CLOCK, the ICP0-Bmal1 interaction may affect viral gene expression. Indeed, it was shown that overexpression of CLOCK partially compensated for the absence of ICP0 in HSV-1 deletion mutants by allowing a higher viral yield and moreover, depletion of CLOCK resulted in a significant decrease in expression of viral proteins. In addition, it was shown that CLOCK is part of a transcriptional complex including the viral proteins ICP4, ICP22 and ICP27 (Kalamvoki and Roizman, 2011). Recently, it was suggested that ICP0 also regulates viral transcription through a specific interaction with the HAT PCAF. Since ICP0 and PCAF were found to co-localize in the ND10s, it was suggested that PCAF improves the ability of ICP0 to activate transcription (Li et al., 2009) (Fig. 6).

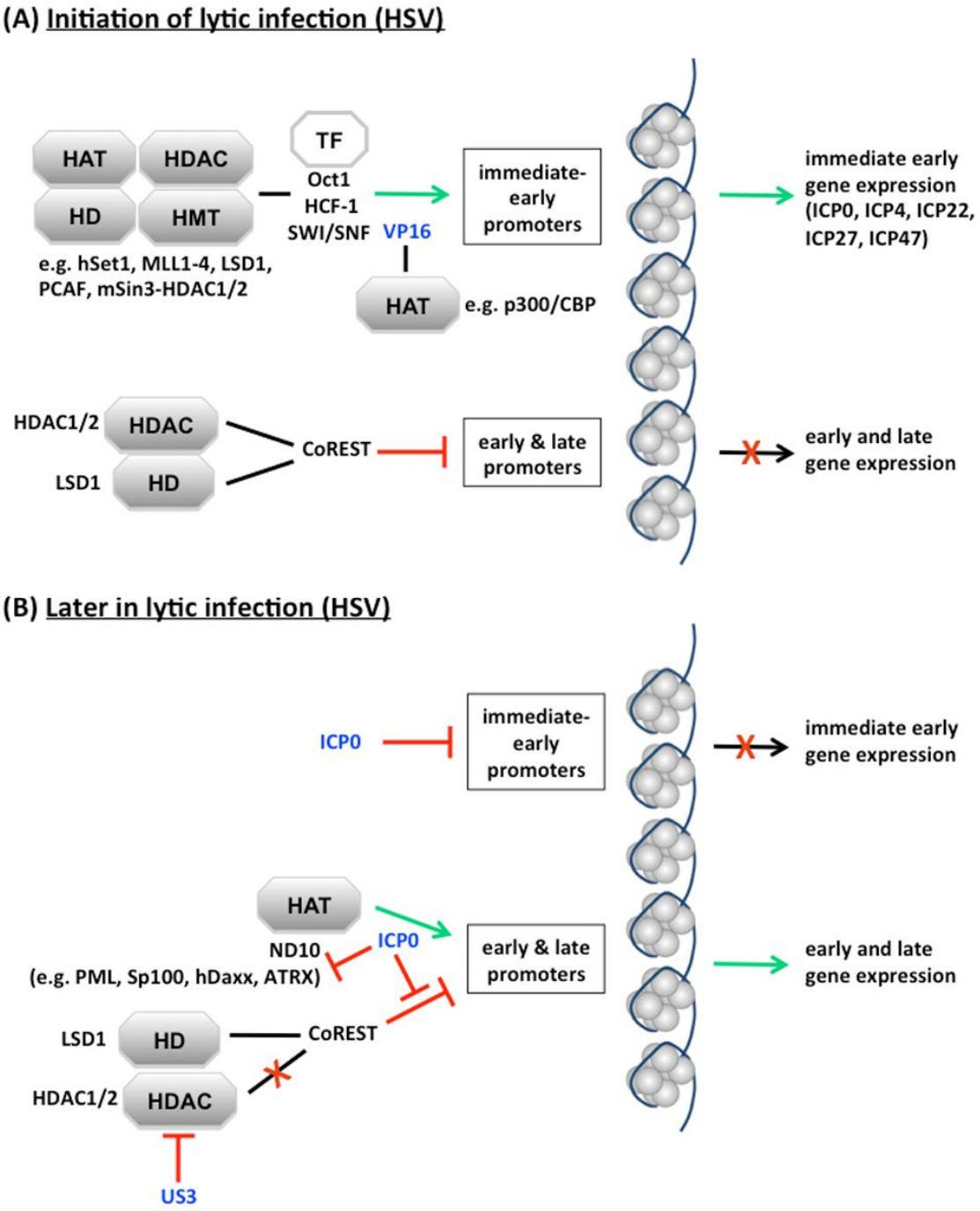


**Figure 6: Some of the major interactions of HSV-1 with histone-modifying enzymes to regulate gene expression during lytic infection.** After initial infection of epithelial cells of the upper respiratory tract, lytic replication starts by recruiting cellular factors to the immediate-early gene promoters. The produced ICP0 will then allow for transcription of the E- and L- genes via two functions: (i) the destruction of ND10s by disrupting PML and (ii) dislocation of HDAC1/2 from the CoREST/REST complex. Here, the major viral proteins and cellular histone-modifying enzymes that are involved are depicted. HDAC: histone deacetylase, HAT: Histone acetyl transferase, HMT: histone methyl transferase, HD: histone demethylase, TF: transcription factor.

Secondly, ICP0 is able to interfere with silencing of viral DNA following immediate-early gene expression by disrupting the function of HDACs (Cliffe and Knipe, 2008). ICP0 directly associates with class II HDACs, but not with class I HDACs when they are present in different histone-modifying complexes (Lomonte et al., 2004). Furthermore, the C-terminal end of ICP0 appears to share a sequence homologous to the N-terminal domain of CoREST. This results in a competition of ICP0 for the binding of HDAC1/2 to the repressive CoREST/REST/HDAC/LSD1 complex and hence, HDAC1/2 become dislodged from this complex in the presence of ICP0 (Gu et al., 2005; Gu and Roizman, 2007) (Fig. 6). The latter was shown to be followed by a phosphorylation of HDAC1 and CoREST and subsequent translocation of these proteins to the cytoplasm. These two activities were found to be independent of ICP0, but to mediated by the viral kinase US3 (Gu et al., 2005; Gu and Roizman, 2007). Another histone modifying enzyme, LSD1, which is also part of the CoREST complex and which has a role in viral gene repression, is partially degraded in a proteasome-dependent manner during a productive HSV-1 infection, although the responsible mechanism for this degradation has not yet been identified (Gu and Roizman, 2009a) (Fig. 6). The effect of ICP0 on promoting viral replication may extend beyond the initial stages of productive infection, and ICP0 might also be involved in reactivation from latency. In a study from Coleman et al. (2008), a fibroblast model of latency was used to illustrate that delivery of ICP0 to quiescent virus genomes resulted in a de-repression, which was characterized by enrichment of acetylated histones on HSV-1 promoters (Coleman et al., 2008). A recent study from Ferenczy & Deluca (2011) showed that ICP0 expression resulted in a decrease in heterochromatin on the whole quiescent genome. High expression of ICP0 was clearly able to facilitate the removal of histones from quiescent viruses, but a low ICP0 expression was not (Ferenczy and Deluca, 2011).

## 1.3 Interferon-mediated antiviral effects and alphaherpesvirus-encoded countermeasures

### 1.3.1 Introduction

Interferons (IFNs) are a group of secreted cytokines that elicit distinct antiviral effects. In this way, IFNs are amongst the key factors of the innate and adaptive immune response against viral infections. They also possess a wide range of other biological activities including regulation of cell growth, differentiation and apoptosis and modulation of immune responses (Samuel, 2001; Meyer, 2009). Interferons are generally grouped into three classes according to their amino acid sequence, designated type I, II and III. Type I IFN is also known as viral IFN and includes IFNα, IFNβ and IFNω. These IFNs are mainly induced by viral infection and can be produced by most infected cell types. However, plasmacytoid dendritic cells (pDCs) are responsible for a large part of the type I IFN production. In mammals, IFNα is encoded by numerous genes (13 in man), while one to three genes encode IFNβ (1 in man). IFNβ appears to be essential for a fully effective antiviral response since IFNα subspecies do not compensate for the loss of IFNβ (Deonarain et al., 2000; Samuel, 2001; Liu, 2005). Type II IFN is referred to as immune IFN or IFNγ, which can only be produced by a certain number of immune cells, especially T-lymphocytes, NK cells, macrophages and conventional dendritic cells (cDCs) (Bach et al., 1997). The most important role for IFNγ is to control long term viral infections as it is a key mediator of the virus-specific cellular immunity (Muller et al., 1994; Cantin et al., 1999). IFNγ is encoded by a single gene (Samuel, 2001). Type III IFNs consist of three IFNλ molecules and represent a class of relatively novel cytokines with biological activities similar to type I IFN. These IFNs seem to have a more specialized role in antiviral defense by exerting host-protection primarily at epithelial surfaces (Ank et al., 2008; Sommereyns et al., 2008; Ank and Paludan, 2009). The type III IFN are coproduced with IFNβ, but act through binding to a different receptor (Meyer, 2009).

The IFN-mediated innate immune system represents the front line of host defence against viral infections. Upon recognition of viral components, both virus-infected cells and immune cells rapidly initiate secretion of IFNs. IFNs do not possess intrinsic antiviral properties, but induce an antiviral state in neighbouring cells that are not yet infected. Binding of the IFNs to their specific receptor leads to the expression of an array of cellular proteins, several of which display antiviral capacities (Samuel, 1991; Samuel, 2001). In the next paragraphs, some of these cellular proteins will be described in detail, followed by a section on alphaherpesviral proteins that counteract the IFN system.

### 1.3.2 Induction of IFN production

During evolution, mammalian cells have evolved a large variety of cellular sensors for viral infection. It is the engagement of these receptors that will eventually lead to the production of type I IFNs. These receptors can be divided into two classes based on their localization. They either associate with cell membranes or are present in the cytoplasm. The difference in localization has an important relevance for the flexibility in triggering type I IFN production, either in infected cells or before cells actually get infected (Garcia-Sastre and Biron, 2006) (Figure 7).

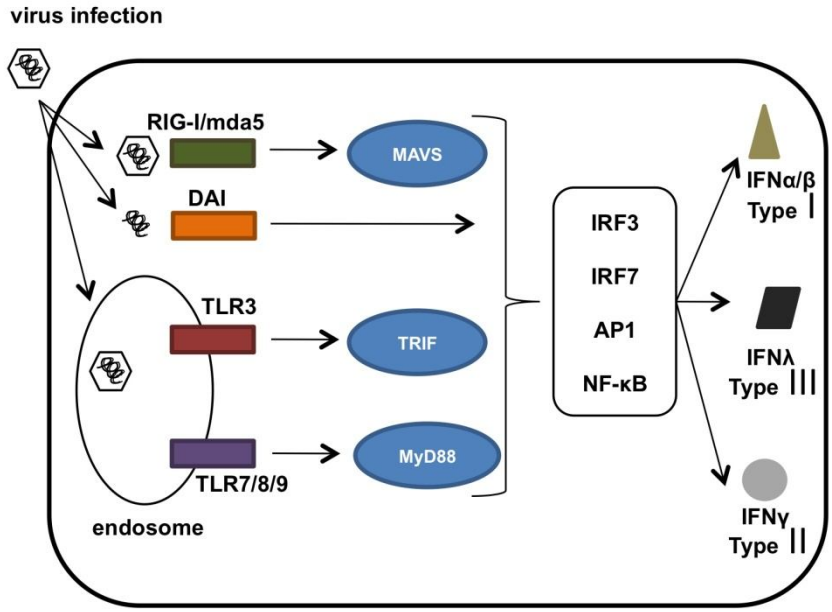


**Figure 7: IFN production pathway (adapted from Deruelle, 2010).**

*Extracytoplasmic pathways for pathogen sensing*

The Toll-like receptor (TLR) family is composed of membrane proteins containing sequences that recognize microbe-associated molecular patterns (MAMPs) in the extracellular environment (Medzhitov and Janeway, 1998; Ausubel, 2005). Upon activation, TLRs transmit signals through their cytoplasmic Toll-interleukin-1 receptor (TIR) domains resulting in the stimulation of gene expression of multiple genes important in both innate and adaptive immunity, including type I IFN (Garcia-Sastre and Biron, 2006). TLR3, TLR7, TLR8 and TLR9 appear to be particularly important in recognizing viral components. TLR3 interacts with dsRNA (most viruses), TLR7 and TLR8 interact with ssRNA (RNA viruses) and TLR9 is important for the recognition of DNA and is as such involved in the antiviral defense against herpesviruses (Hemmi et al., 2000; Alexopoulou et al., 2001; Diebold et al., 2004; Heil et al.,

2004; Lund et al., 2004; Akira et al., 2006). These TLRs are localized in endosomal compartments and contact with viral components occurs through endosomal-mediated internalization of virus or viral material. To allow this contact, viral degradation in the endosome is required (Garcia-Sastre and Biron, 2006).

Signalling is different for TLR3 versus TLR7/8/9. In response to dsRNA, TLR3 recruits a TIR-domain containing adapter protein called TRIF which is able to indirectly activate numerous transcription factors, including IFN regulatory factor 3 (IRF3) which, when phosphorylated, induces the expression of type I IFN. NF-κB and activating protein 1 (AP1) are also stimulated by TRIF resulting in regulation of IFNβ production (Kawai and Akira, 2006; Randall and Goodbourn, 2008) (Fig.7). Upon nucleic acid recognition, TLR7, TLR8 and TLR9 recruit a TIR-domain containing adapter protein called MyD88, which is used by all TLRs except TLR3, leading to the activation of NF-κB. The type I IFN production upon TLR7/8/9 is independent of IRF3 but occurs through another member of the family, IRF7 (Fig.7). IRF7 is structurally very similar to IRF3 but is able to activate both IFNα and IFNβ promoters (Marie et al., 1998; Kawai and Akira, 2006; Randall and Goodbourn, 2008). TLR7/8/9 and IRF7 are constitutively expressed in pDCs correlating with the ability of this particular cell type to rapidly synthesize massive amounts of type I IFNs (Kato et al., 2005; Garcia-Sastre and Biron, 2006). Type III IFNs can also be induced by activation of TLRs as the promoters of type III IFN genes share similarities with type I IFN genes and both are regulated by IRF3 and IRF7 (Ank et al., 2008; Ank and Paludan, 2009).

*Cytoplasmic pathways for pathogen sensing*

TLRs recognize pathogens either at the cell surface or in endosomal compartments, indicating that the TLR system is not used for the detection of pathogens that already have invaded the cytosol (Akira et al., 2006). Fibroblasts and cDCs lacking MyD88 and TRIF are still capable of inducing type I IFN upon viral infection. This indicates that the TLR system is not required for viral detection in several cell types (Akira et al., 2006). The TLR-independent cytosolic pathways of viral recognition can be subdivided in two categories: RNA recognition receptors and DNA recognition receptors (Takaoka and Taniguchi, 2008). The first category, the RNA recognition receptors, consists of members of a family of DExD/H box RNA helicases which contain a caspase-recruiting domain (CARD) (Yoneyama et al., 2004). So far, two such sensors have been described: the retinoic acid-inducible gene I (RIG-I) and the melanoma differentiation-associated gene 5 (mda5) (Kang et al., 2002; Andrejeva et al., 2004; Yoneyama et al., 2004) (Fig.7). Both helicases interact through their CARD domain with the CARD-like domain of the mitochondrial antiviral signalling protein (MAVS), which is

also called IFNβ promoter stimulator 1 (IPS1), virus-induced signalling adaptor (VISA) or CARD adaptor-inducing IFNβ (CARDIF) (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). The binding between RIG-I and one of these RIG-I-like receptors (RLRs) results in the activation of IRF3, NF-κB and AP1, subsequently leading to type I IFN production (Akira et al., 2006; Garcia-Sastre and Biron, 2006; Takeuchi and Akira, 2009). RLRs also seem to stimulate expression of type III IFN since ectopic expression of a constitutively active form of RIG-I enhances expression of both IFNβ and IFNλ (Matikainen et al., 2006).

The second category of cytosolic recognition factors are the DNA recognition receptors. These are involved in the initiation of an innate immune response against viral dsDNA, like herpesviruses. The first real DNA-sensing molecule that was identified is the ZBP1/DLM-1 or DNA-dependent activator of IRFs (DAI) (Fig.7). This protein is able to interact with DNA, leading to activation of IRF transcription factors for type I IFN gene induction (Takaoka et al., 2007). It was shown that IRF3 is the major transcription factor to induce type I IFN in response to cytoplasmic DNA (Takaoka et al., 2007). Most of these studies were performed with synthetic DNA. However, more recently, ZBP1 was implicated in the activation of type I IFN upon HCMV infection (Defilippis et al., 2010). STING (stimulator of interferon genes) is an endoplasmic reticulum resident transmembrane protein that has been shown to facilitate production of type I IFNs (Ishikawa and Barber, 2008). The same group demonstrated that STING may be essential for the recognition of intracellular herpesvirus DNA, leading to the production of IFN, as the loss of STING rendered mice susceptible to lethal HSV infection (Ishikawa et al., 2009). More recently, yet another cytoplasmic DNA sensor was discovered. The IFI16 protein is a member of the PYHIN protein family that contains a pyrin domain and two DNA-binding HIN domains and was found to interact with transfected DNA to induce IFNβ through recruitment of STING. Therefore PHYIN proteins are proposed to be a new family of innate DNA sensors called ‘AIM2-like receptors’ (ALRs) (Unterholzner et al., 2010).

### 1.3.3 Signal transduction in response to IFNs

The biological activities of IFNs are initiated by the binding of IFNs to their cognate receptors on the surface of the cell, resulting in the activation of the Jak/STAT pathways (reviewed in Goodbourn et al., 2000; Samuel, 2001; Randall and Goodbourn, 2008) (Figure 8).

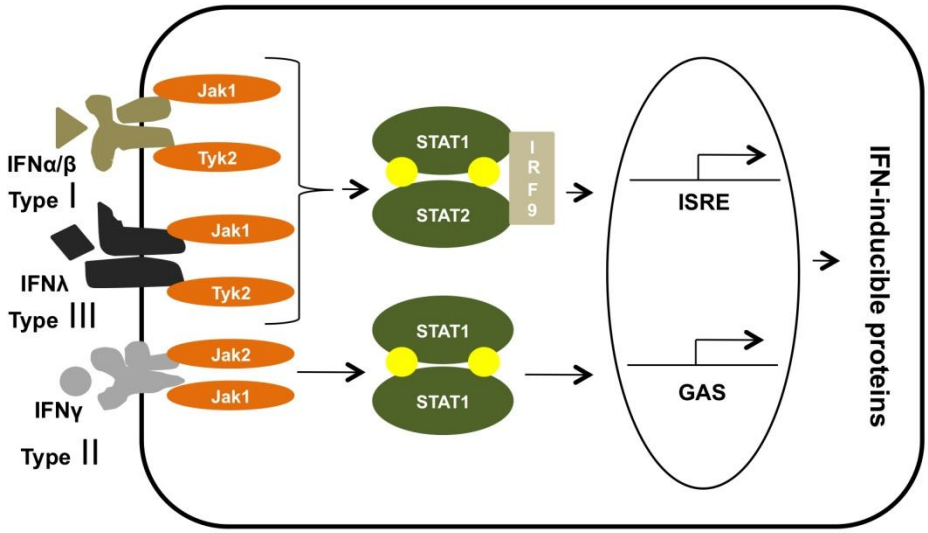


**Figure 8: Signal transduction in response to IFNs (adapted from Deruelle, 2010).**

All type I IFNs share a common heterodimeric receptor composed of IFNAR1 and IFNAR2 (reviewed in (Mogensen et al., 1999). Prior to stimulation of the receptor, the cytoplasmic domains of IFNAR1 and IFNAR2 are associated with the 'Janus' tyrosine kinase Tyk2 and Jak1, respectively (Colamonici et al., 1994; Novick et al., 1994). IFNAR2 is also bound to STAT2 that weakly interacts with STAT1 (Li et al., 1997). Ligand-induced dimerization of both parts of the receptor causes a conformational change, resulting in the phosphorylation of tyrosine 466 ($Tyr^{466}$) of IFNAR1 by Tyk2 thereby creating a docking site for STAT2 (Colamonici et al., 1994; Novick et al., 1994; Yan et al., 1996). Subsequently, STAT2 is phosphorylated by Tyk2 on $Tyr^{690}$ while STAT1 is phosphorylated by Jak1 on $Tyr^{701}$ allowing the formation of a stable heterodimer (Shuai et al., 1993; Leung et al., 1995; Qureshi et al., 1996). The latter creates a novel NLS and phosphorylation of STAT2 inactivates the dominant constitutive nuclear export signal of STAT2, resulting in the nuclear transport of the dimer where it remains until dephosphorylation occurs (Banninger and Reich, 2004; Frahm et al., 2006; Reich and Liu, 2006). The STAT1-STAT2 complex associates with IRF9 to form a heterotrimer ISGF3 complex. This ISGF3 complex is able to bind to the IFN-stimulated response element (ISRE) present in the promoters of most IFN-inducible genes (Goodbourn et al., 2000; Randall and Goodbourn, 2008). Before, it was generally accepted

that the assembly of ISGF3 took place in the nucleus, but more recently it was shown that assembly is co-ordinated at the receptor (Tang et al., 2007). Upon stimulation of IFNAR2, the transcriptional co-factor CBP is recruited causing the acetylation of IFNAR2, which creates a docking site for IRF9. The latter as well as STAT1 and STAT2 will subsequently also undergo acetylation, which may aid the formation of the ISGF3 complex (Tang et al., 2007).

Type III IFN signalling follows a highly similar pattern as type I IFN although a different receptor is engaged (Zhou et al., 2007). The type III IFN receptor consists of the IL10R2 chain and the IFNλ-specific IL28Rα chain (also called IFNLR1). The latter receptors are only expressed in epithelial cells and pDCs (Ank and Paludan, 2009).

Type II IFN differs somewhat from this signalling pathway. Like the other IFN receptors, the constitutive type II IFN receptor also consists of a heterodimeric glycoprotein, composed of IFNGR1 and IFNGR2. These latter are weakly pre-associated in unstimulated cells (Bach et al., 1996). The cytoplamsic domains of the receptors are linked to Jak1 and Jak2, respectively (Kotenko et al., 1995; Sakatsume et al., 1995; Bach et al., 1996; Kaplan et al., 1996). Upon IFNγ binding, the receptors undergo dimerization, triggering the signalling pathway (Greenlund et al., 1994; Igarashi et al., 1994; Greenlund et al., 1995; Bach et al., 1996). As a consequence of dimerization, Jak2 is activated, which *trans*-phosphorylates and activates Jak1 (Briscoe et al., 1996). The activated Jaks then phosphorylate a tyrosine-containing sequence near the C-terminus of IFNGR1, thereby forming paired binding sites for STAT1 (Greenlund et al., 1994; Greenlund et al., 1995). Two STAT1 molecules are then able to interact with IFNGR1 and are phosphorylated at $Tyr^{701}$, resulting in their activation and dissociation from the receptors (Shuai et al., 1993; Shuai et al., 1994). The active STAT1 molecules form a homodimer, which translocates to the nucleus and binds the gamma-activation sequence (GAS), thereby stimulating transcription of specific interferon-stimulated genes (ISGs) (Sekimoto et al., 1996; Stark et al., 1998). It is important to notice that the GAS is different from the ISRE and that the binding of the STAT1 complex does not require IRF9 (Goodbourn et al., 2000; Randall and Goodbourn, 2008).

It has also been reported that STAT1 homodimers may activate type I IFN signalling and trigger transcription of some type I IFN-regulated genes, but the overall significance in antiviral response is unknown (Decker et al., 1991; Randall and Goodbourn, 2008).

### 1.3.4 Interferon-mediated antiviral effects

Treatment of cells with type I IFN results in the stimulation of expression of hundreds of genes, several of which are involved in the establishment of the so-called antiviral state of the cell. Several of these genes encode enzymes, e.g. dsRNA-dependent protein kinase R (PKR), 2'5'-oligoadenylate synthetase (OAS) and Mx (reviewed in Goodbourn et al., 2000; Samuel, 2001; Randall and Goodbourn, 2008). Figure 9 shows a schematic overview of the IFN-inducible products as described below.

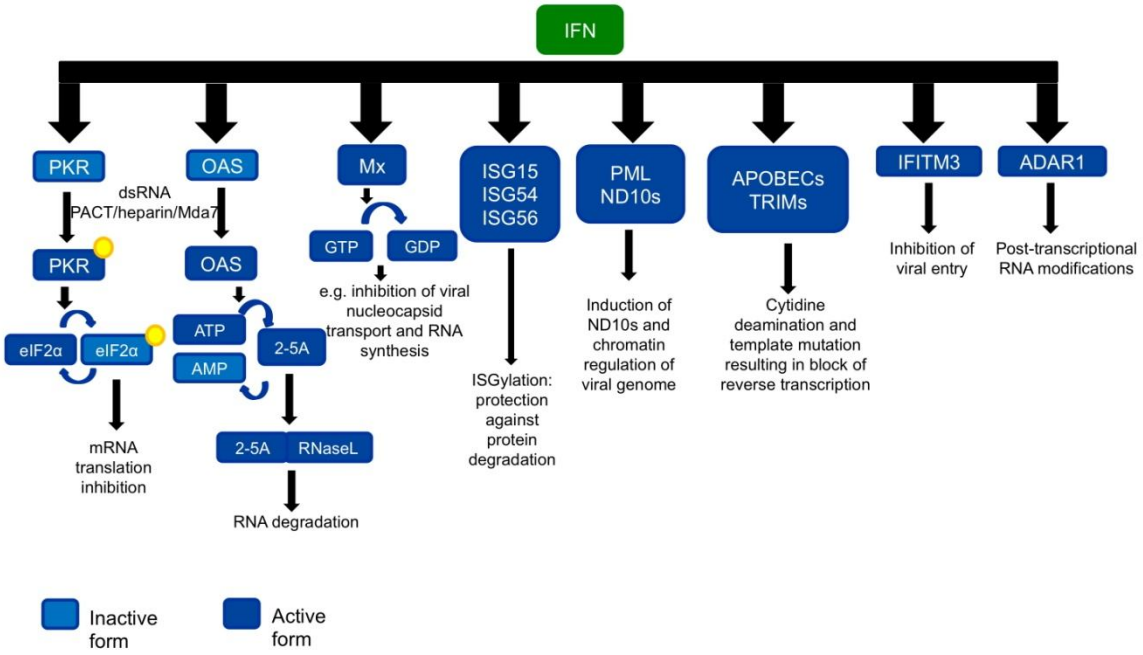


**Figure 9: overview of IFN-inducible antiviral proteins (adapted from Deruelle, 2010).**

*dsRNA-dependent protein kinase R (PKR)*

The IFN-inducible PKR is a serine/threonine kinase with multiple functions in control of transcription and translation (reviewed in Clemens and Elia, 1997). PKR is synthesized in an inactive form and, in response to the cofactor dsRNA produced during viral infection, undergoes a conformational change resulting in dimerization and activation (Meurs et al., 1990; Katze et al., 1991; George et al., 1996). Alternatively, PKR may be activated by a stress-activated protein called protein kinase R (PKR)-activating protein (PACT) (Ito et al., 1999; Patel et al., 2000). One of the best characterized PKR substrates is the α subunit of the eukaryotic translation initiation factor 2 (eIF2α). Phosphorylated eIF2α prevents the recycling of eIF2α such that translation initiation is halted (Meurs et al., 1992; Meurs et al., 1993). A variety of physiologic conditions, including the combination of IFN and virus

infection, cause the phosphorylation state of eIF2α to increase and a subsequent suppression of mRNA translation. In the initial step of translation, the initiator Met-tRNA is recruited to the 40S ribosomal subunit via an interaction with GTP-bound eIF2α. This complex then interacts with mRNA, other initiator factors and the large ribosomal subunit to form a pre-initiator complex, with subsequent hydrolysis of the GTP molecule bound to eIF2α and release of GDP-bound eIF2α. To allow to participate in another round of translation initiation, the GDP molecule bound to eIF2α must be exchanged for GTP. This is catalysed by the guanine exchange factor eIF2β. Phosphorylated eIF2α strongly interacts with eIF2β and traps it such that it cannot mediate recycling of eIF2α. Since eIF2β is present in limiting amounts, translation is inhibited (Ramaiah et al., 1994; Clemens and Elia, 1997; Goodbourn et al., 2000; Randall and Goodbourn, 2008).

PKR, in response to dsRNA, is also involved in the activation of NF-κB, which is essential for induction of IFNβ production. It has also been suggested that PKR influences the activity of the transcription factors STAT1, IRF1 and p53 (Kumar et al., 1997; Wong et al., 1997; Cuddihy et al., 1999a; Cuddihy et al., 1999b; Ramana et al., 2000). PKR can also aid the clearance of viruses by mediating apoptosis (Der et al., 1997; King and Goodbourn, 1998; Tanaka et al., 1998; Goodbourn et al., 2000).

Although there is abundant evidence that PKR plays a major role in regulating virus infection, PKR is not sufficient to mediate the full antiviral response. Evidence for this came from PKR knockout mice that still show IFN-mediated resistance against viral infection (Yang et al., 1995; Abraham et al., 1999; Goodbourn et al., 2000).

*2’5’-oligoadenylate synthetase (OAS) and RNaseL*

The 2’5’-oligoadenylate synthetases are a group of enzymes that are induced by IFNs in mammalian cells. They catalyze the synthesis of adenosine oligomers linked by phosphodiester bonds in the unusual conformation of 2’ to 5’ (Kerr and Brown, 1978). Like PKR, OAS is synthesized in an inactive form and utilizes dsRNA as cofactor. The 2’5’ adenosine molecules bind with high affinity to endoribonuclease L (RNaseL) and induce its activation by dimerization. Active RNaseL catalyses the cleavage of ssRNA, including mRNA. This may lead to inhibition of protein synthesis (Imai et al., 1982; Silverman, 1997). It has also been shown that RNaseL is able to cleave 28S ribosomal RNA in a site-specific manner resulting in ribosomal inactivation and thus translational inhibition (Goodbourn et al., 2000; Iordanov et al., 2000). Since OAS is very labile, the activation of RNaseL relies on locally activated OAS within the cell, thus ensuring that viral transcripts are destroyed preferentially over cellular mRNAs (Nilsen and Baglioni, 1979; Goodbourn et al., 2000).

*Mx*

Mx and the Mx family of genes encode large GTPases related to dynamin. The GTPase activity appears to be essential for their antiviral functions (Pitossi et al., 1993). They exert an antiviral effect against a wide range of RNA viruses. The Mx GTPases probably interfere with virus replication by inhibiting viral polymerases (Stranden et al., 1993). The Mx GTPases are also able to recognize nucleocapsid-like structures and disturb their localization within the cell, resulting in a restricted virus replication (Kochs et al., 1998; Goodbourn et al., 2000; Weber et al., 2000; Randall and Goodbourn, 2008).

*ISG15, ISG54 and ISG56*

Several genes are upregulated in response to IFN and were named according to their molecular weight. For example, ISG15 is a 15kDa protein induced by IFN. Of all the IFN-stimulated genes, ISG15 is the most abundant but little is known about its function (reviewed by Kerscher et al., 2006). ISG15 modification, called ISGylation, occurs on over 100 cellular proteins and is catalyzed by the action of IFN-inducible E1, E2 and E3 ubiquitin ligases. Unlike ubiquitination, ISGylation is an IFN-stimulated and regulated process that appears to mimic mono-ubiquitination functions, such as modulation of enzymatic activity, rather than the poly-ubiquitination function of protein degradation (Harty et al., 2009). It has been shown that ISGylation inhibits degradation of proteins rather than stimulating it like ubiquitination (Sadler and Williams, 2008; Durfee et al., 2010; Liu et al., 2010; Shi et al., 2010). Many of the substrates for ISG15 play important roles in the innate immunity including Jak1, Erk1, STAT1, PKR, MxA, RIG-I and IRF3 (Malakhov et al., 2003; Zhao et al., 2005; Lu et al., 2006; Okumura et al., 2007; Randall and Goodbourn, 2008). Interestingly, ISG15 is also released from IFN-treated cells. Several activities have been attributed to recombinant or extracellular ISG15 including an induction of NK cell proliferation, augmented lymphokine-activated-killer (LAK) activity, increased production of IFN-γ, maturation of DC, chemotactic activity towards neutrophils. To date, no cell surface receptor has been identified that binds to ISG15; therefore the role that its potential cytokine activity plays *in vivo* is yet to be ascertained (D'cunha et al., 1996; Padovan et al., 2002; Owhashi et al., 2003; Lenschow, 2010).

ISG54 and ISG56 are related proteins that inhibit translation by interacting with the multiprotein translation initiation factor eIF3 complex thereby inhibiting its ability to stabilize the eIF2α-GTP-tRNA complex (Hui et al., 2003; Randall and Goodbourn, 2008).

*Promyelocytic leukaemia (PML) nuclear bodies*

The role of PML nuclear bodies in the antiviral response has been extensively studied (Everett and Chelbi-Alix, 2007). These PML bodies or ND10 bodies contain, amongst others, IFN-inducible PML and Sp100. They play an important role in transcriptional responses to stress and regulate chromatin structure and thus gene promoter accessibility. The depletion of PML enhances growth of herpesviruses, indicating that ND10s do play a role in restricting viral replication (Randall and Goodbourn, 2008).

*RNA editing proteins*

Several 'restriction factors' that are able to restrict replication of retroviruses have been identified. These are proteins that control normal cell functions and are under positive selection pressure as they are evolving rapidly. The best characterized example is apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like (APOBEC) 3F and 3G genes (APOBEC3F and APOBEC3G) and the unrelated tripartite motif-α (TRIM5α) gene. Although they are expressed constitutively, they are strongly upregulated by type I IFNs in all cell types (Asaoka et al., 2005; Bonvin et al., 2006; Chen et al., 2006; Peng et al., 2006; Sarkis et al., 2006; Argyris et al., 2007; Sakuma et al., 2007; Ying et al., 2007). The universal mechanism used by these proteins involves RNA editing through cytidine deamination and subsequent mutation of the viral RNA template (Baumert et al., 2007; Randall and Goodbourn, 2008). It was recently shown that APOBEC3C is able to reduce both virus titers and particle/PFU ratio in tissue cultures infected with HSV-1 (Suspene et al., 2011).

Another important RNA-editing enzyme, called ADAR1, is induced by IFN and acts as an RNA-specific adenosine deaminase (George and Samuel, 1999). It was described that ADAR1 is involved in the editing of viral RNA transcripts and cellular pre-mRNAs (Bass, 1997; Samuel, 2001).

*IFITM*

The interferon induced transmembrane (IFITM) proteins 1, 2 and 3 were recently identified as host factors that are able to restrict viral entry (Brass et al., 2009; Liu et al., 2010). Knockdown of IFITM3 or deletion of the *Ifitm* locus increased susceptibility of the cells to viral infections, although the exact mechanism remains unknown (Yount et al., 2010).

### 1.3.5 Alphaherpesvirus-encoded countermeasures to the IFN response

There are five main ways by which viruses circumvent the IFN response: i) interfering globally with host-cell gene expression and/or protein synthesis; ii) minimizing IFN induction by limiting production of MAMPs and/or by specifically blocking IFN-induction cascades; iii) inhibiting IFN signalling; iv) blocking the action of IFN-induced enzymes with antiviral effects and v) having a replication strategy that is largely insensitive to IFN (Randall and Goodbourn, 2008). Different viruses have evolved a great diversity of mechanisms to achieve similar ends to counteract the IFN response. A combination of the above mentioned strategies might be necessary to sufficiently circumvent IFN-mediated antiviral effects to allow replication and spread. Figure 10 shows schematically where the HSV-1-encoded viral proteins interact with the IFN production or signalling pathway. An overview of all alphaherpesvirus-encoded IFN-antagonists is given below (Table 1).

Several viral proteins of HSV-1 have been described to have antagonistic effects on IFN production, mainly by targeting IRF3 (belonging to strategy ii) mentioned higher). The activated IRF3 is sequestered by **ICP0** together with its co-activator CBP/p300, followed by inactivation and degradation of IRF3 hence blocking IFNβ production (Melroe et al., 2007). It has also been shown that ICP0 inhibits IRF7 through its RING finger domain (Lin et al., 2004). In addition to ICP0, the endoribonuclease **vhs** protein of HSV-1 was found to non-specifically degrade ISG transcripts that accumulate in the absence of ICP0 (Lin et al., 2004). Recently, it was described that infection with a vhs deficient virus induces more IFN than WT virus again indicating that vhs functions to reduce innate immune responses by reducing levels of dsRNA (Pasieka et al., 2008). **ICP27** is also able to inhibit IRF3 activation and NF-κB in human macrophages and DCs (Melchjorsen et al., 2006). In contrast, in a primary human fibroblast cell line, infection with a ICP27-null mutant did not result in an observable ISG induction and no increase in IRF3 activity was found, indicating that the involvement of ICP27 in IRF3 inhibition may be cell type dependent (Lin et al., 2004; Melroe et al., 2004; Paladino and Mossman, 2009). Another protein of HSV-1, **US3**, has been suggested to downregulate the TLR3-mediated response, preventing IRF3 activation (Peri et al., 2008). Recently, it was shown that **ICP34.5** can inhibit IRF3 activation through binding of TBK1 (Verpooten et al., 2009).

Next to HSV-1, the BoHV-1 protein **bICP0**, was shown to inhibit the kinases TBK1 and IKKi, leading to inhibition of IRF3 and thus IFN production (Henderson et al., 2005).

Also for VZV, it has been described that production of type I IFN can be reduced through inhibition of the NF-κB pathway, but through a still unknown mechanism (Jones and Arvin, 2006). Recently, it was shown that the VZV protein **ORF47** is able to bind IRF3, which prevents its homodimerization and subsequent induction of ISG (Vandevenne et al., 2011). Another recent study showed that **ORF61** causes degradation of activated IRF3 (Zhu et al., 2011).

Alphaherpesviruses have also been reported to interfere with IFN signalling, typically by blocking the Jak/STAT signalling pathway, thereby inhibiting production of ISG (strategy iii). Inhibition of Jak1, Tyk2, STAT1 and STAT2 phosphorylation during the initial phases of infection with HSV-1 was reported in early studies (Yokota et al., 2001). This inhibition of Jak/STAT phosphorylation correlated with the induction of suppressor of cytokine signalling 3 (SOCS3), which is a negative regulator of IFN signalling, and the viral proteins **vhs** and **UL13** were found to be involved (Yokota et al., 2001; Yokota et al., 2004). Chee and Roizman subsequently showed that vhs was at least partly responsible for the decline in Jak/STAT protein members (Chee and Roizman, 2004). More recently, it was reported that HSV-1 is able to interfere with IFNγ induced STAT1 signalling in infected mature DCs due to downregulation of IFNGR1 and inhibition of STAT1 phosphorylation. It was suggested that vhs again plays an important role in this process (Eisemann et al., 2007). **ICP27** can also downregulate STAT1 phosphorylation upstream of Jak1 activation (Johnson et al., 2008; Johnson and Knipe, 2010). In addition, Liang and Roizman reported that **US3** phosphorylates a subunit of the IFNγ receptor, thereby inhibiting downstream signalling (Liang and Roizman, 2008). An earlier report suggested that US3 is involved in overcoming the IFN-induced antiviral state since, based on plaque number and size, a US3null HSV-1 virus was found to be more sensitive to IFNα compared to WT virus (Piroozmand et al., 2004).

For VZV, it was described that expression of STAT1 and Jak2 are inhibited, while Jak1 expression levels remain unaltered (Samuel, 2001). Expression of IRF1 and MHCII transactivator (CIITA), which are regulated by IFNγ, are also inhibited by VZV (Abendroth et al., 2000). The VZV orthologue of US3, **ORF66**, decreases the level of STAT1 phosphorylation following IFNγ exposure (Schaap et al., 2005).

For PRV, it has been shown that the IFN-mediated immune response can be counteracted by a decreased phosphorylation of STAT1. Brukman and Enquist showed that structural components of the virion are sufficient to mediate this, although the exact mechanism remains undetermined (Brukman and Enquist, 2006b).

Alphaherpesviruses can also antagonize the IFN-inducible antiviral proteins (strategy iv). **ICP0** of HSV-1 is able to interact with the proteasome-dependent degradation pathway leading to a destabilisation of certain ISG products, including PML (Maul et al., 1993; Maul and Everett, 1994; Chelbi-Alix and De The, 1999; Chelbi-Alix and De Thé, 1999; Parkinson and Everett, 2000; Eidson et al., 2002). The RING finger domain of ICP0 is required for this proteasomal degradation as it acts as a ubiquitin E3 ligase (Everett and Maul, 1994; Maul and Everett, 1994; Boutell et al., 2002). In addition, HSV-1 encodes two proteins that interfere with the PKR pathway: **ICP34.5** and **US11**. ICP34.5 acts through the recruitment of the cellular protein phosphatase 1 (PP1), forming a high-molecular weight complex that efficiently dephosphorylates eIF2α (He et al., 1997; He et al., 1998). Recently, it was shown that ICP34.5 functions as a bridge between PP1 and eIF2α as both proteins are able to directly bind ICP34.5, thereby allowing the proteins to be in each other's vicinity (Li et al., 2011). The **US11** protein alters the PKR pathway in a different way. US11 interacts with dsRNA directly to inhibit PKR phosphorylation and activation, thereby preventing eIF2α phosphorylation (Poppers et al., 2000; Sanchez and Mohr, 2007). Both proteins are required for virus replication in IFN-treated cells as their combined action prevents the accumulation of phosphorylated eIF2α (Mulvey et al., 2003; Mulvey et al., 2004).

For VZV, it was described that **IE63**, orthologue of ICP22 of HSV-1, is required and sufficient to inhibit phosphorylation of eIF2α but the underlying mechanism still remains unknown (Ambagala and Cohen, 2007).

PRV encodes **EP0**, an orthologue of ICP0 of HSV-1, which has been described to counteract the IFN-induced antiviral state in primary pig cells, but not in non-host cells (Brukman and Enquist, 2006a).

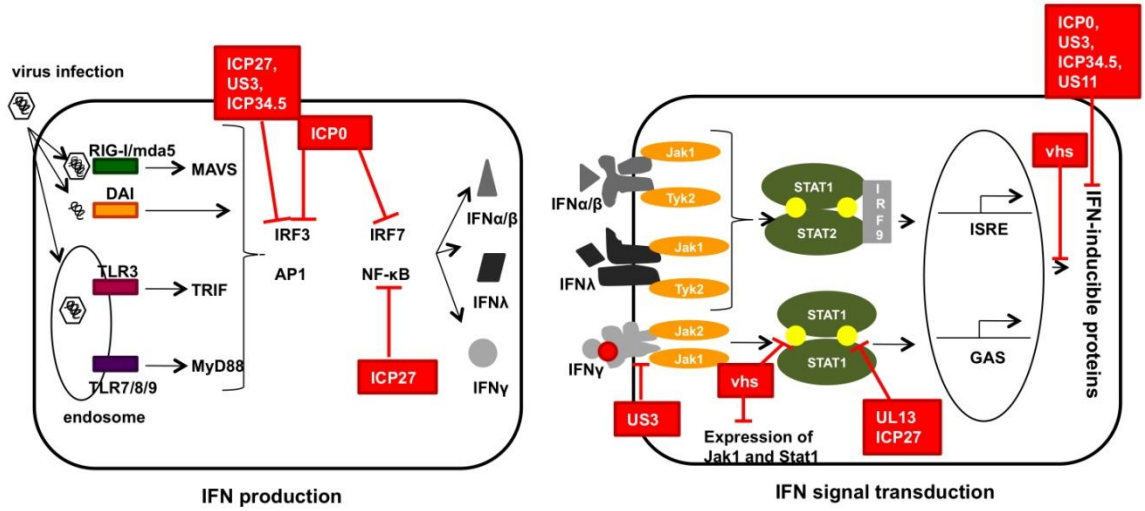


**Figure 10: Overview of the viral proteins encoded by Herpes Simplex Virus that counteract the interferon system.**

Table 1: Overview of viral proteins that counteract IFN-mediated antiviral effects

| Virus | Protein | Function | Reference |
|---|---|---|---|
| HSV-1 | ICP0 | Inhibition of IRF3 | Melroe et al. 2007 |
| | | Inhibition of IRF7 | Lin et al., 2004 |
| | | Degradation of IFN-inducible gene products e.g. PML | Maul and Everett, 1994 |
| | Vhs | Degradation of ISG transcripts | Lin et al., 2004 |
| | | Decline in Jak/STAT protein levels | Chee and Roizman, 2004 |
| | | Inhibition of STAT1 phosphorylation | Eisemann et al., 2007 |
| | UL13 | Inhibition of Jak/STAT phosphorylation | Yokota et al., 2004 |
| | ICP27 | Inhibition of IRF3 and NF-κB | Melchjorsen et al., 2006 |
| | | Downregulate STAT1 phosphorylation | Johnson et al., 2008 |
| | US3 | Phosphorylation of IFNγ receptor subunit | Liang and Roizman, 2008 |
| | | Preventing IRF3 activation | Peri et al., 2008 |
| | | Overcomes an IFN-induced antiviral state | Piroozmand et al., 2004 |
| | ICP34.5 | Inhibition of IRF3 through binding TBK1 | Verpooten et al., 2009 |
| | | Recruitment of PP1 to dephosphorylate eIF2α | He et al., 1997 |
| | US11 | Inhibits PKR activation, preventing phosphorylation of eIF2α | Poppers et al., 2000 |
| BHV-1 | bICP0 | Inhibition of TBK1 and IKKi, leading to inhibition of IRF3 | Henderson et al., 2005 |
| VZV | ORF47 | Binding to IRF3 and prevent homodimerization | Vandevenne et al., 2011 |
| | ORF66 | Decrease phosphorylation of STAT1 | Schaap et al., 2005 |
| | IE63 | Induces dephosphorylation of eIF2α | Ambagala and Cohen, 2007 |
| | ORF61 | Degradation of activated IRF3 | Zhu et al., 2011 |
| PRV | EP0 | Overcomes an IFN-induced antiviral state | Brukman and Enquist, 2006 |
| | ? | Decrease phosphorylation of STAT1 | Brukman and Enquist, 2006 |

?: unidentified viral protein

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## Chapter 2: Aims

Herpesviruses are among the most successful pathogens, establishing lifelong latent infections from which they may reactivate to spread to new hosts. Key to this success is their delicate and complex balance with the host immune system. Interferon is one of the major components of the host defense against viruses in general and herpesviruses in particular. Indeed, defects in the interferon system, particularly the type I interferon system, may lead to aggravated and sometimes fatal herpesvirus disease (Al-Khatib et al., 2002; Al-Khatib et al., 2003; Vollstedt et al., 2004; Casrouge et al., 2006; Zhang et al., 2007; Zhang et al., 2008; Perez De Diego et al., 2010).

The general aim of the current thesis was to gain a better insight in the interaction of herpesviruses with the interferon system. Our studies focussed on the porcine alphaherpesvirus pseudorabies virus and the closely related subfamily human member of this subfamily, the herpes simplex virus.

A first aim of this study was to investigate, using an *in vitro* system, whether type I interferon may play a role in the establishment of PRV and HSV-1 latency in sensory neurons (Chapter 3).

To further dissect the role for IFN in latency establishment, we next investigated the effect of IFN on immediate-early gene transcription and translation, both for PRV (IE180 gene) and HSV-1 (ICP4 gene) (Chapter 4).

Further, we investigated whether part of the suppressive effect of IFN on expression of the IE gene ICP4 of HSV may be through a previously uncharacterized effect of IFN on histone modifications (Chapter 5).

As a countermeasure towards the suppressive effects of IFN on viral replication, herpesviruses have developed systems to interfere with the antiviral effects of IFN. In a fourth experimental chapter, we investigated how PRV blocks phosphorylation of eIF2α, one of the most potent antiviral effects of IFN (Chapter 6).

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# Chapter 3: Interferon Alpha Induces Establishment of Alphaherpesvirus Latency in Sensory Neurons *In Vitro*

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## Abstract

BACKGROUND: Several alphaherpesviruses, including herpes simplex virus 1 (HSV-1) and pseudorabies virus (PRV), establish lifelong latency in neurons of the trigeminal ganglion (TG). Although it is thought that efficient establishment of alphaherpesvirus latency is based on a subtle interplay between virus, neurons and the immune system, it is not clear which immune components are of major importance for the establishment of latency. METHODOLOGY/PRINCIPAL FINDINGS: Here, using an *in vitro* model that enables a natural route of infection, we show that interferon alpha (IFNα) has the previously uncharacterized capacity to induce a quiescent HSV-1 and PRV infection in porcine TG neurons that shows strong similarity to in vivo latency. IFNα induced a stably suppressed HSV-1 and PRV infection in TG neurons *in vitro*. Subsequent treatment of neurons containing stably suppressed virus with forskolin resulted in reactivation of both viruses. HSV and PRV latency *in vivo* is often accompanied by the expression of latency associated transcripts (LATs). Infection of TG neurons with an HSV-1 mutant expressing LacZ under control of the LAT promoter showed activation of the LAT promoter and RT-PCR analysis confirmed that both HSV-1 and PRV express LATs during latency *in vitro*. CONCLUSIONS/SIGNIFICANCE: These data represent a unique *in vitro* model of alphaherpesvirus latency and indicate that IFNα may be a driving force in promoting efficient latency establishment.

## 1. Introduction

Alphaherpesviruses are a subfamily of the herpesviruses containing closely related human and animal pathogens, including human HSV-1 (cold sores, corneal blindness, and encephalitis) and important animal viruses such as the porcine pseudorabies virus (PRV) and bovine herpesvirus 1 (BoHV-1; respiratory symptoms, abortions, and/or neurological symptoms).

Cycles of latency and reactivation arguably constitute the most important and fascinating hallmarks of alphaherpesvirus infections. Alphaherpesviruses generally establish latency in sensory neurons, and neurons of the trigeminal ganglion (TG) are the predominant site of latency for several important alphaherpesviruses, such as HSV-1, PRV, and BoHV-1 (Croen et al., 1987; Gutekunst et al., 1980; Jones, 2003). Although there is direct and indirect evidence to support the general concept that alphaherpesvirus latency and reactivation is based on a subtle interplay between virus, neurons and the immune system, many questions remain about the immune components that are involved in the establishment of latency (Decman et al., 2005).

It is becoming increasingly clear that the innate immune system has an important role in controlling alphaherpesvirus infections. Type I interferons (IFNα and -β) are among the first immune effectors produced upon alphaherpesvirus infection (Jones et al., 2003; Mikloska et al., 1998) and it has been shown that they are important in limiting viral replication and spread in vitro, but also in vivo at the periphery during initial infection and during reactivation (Hendricks et al., 1991; Mikloska & Cunningham, 2001; Sainz & Halford, 2002). Furthermore, type I interferons have been shown to be present at the periphery (Hendricks et al., 1991) and within the ganglion (Carr et al., 1998) around the time point that latency is established.

In the current study, using an *in vitro* two-chamber model that enables a natural route of alphaherpesvirus infection of porcine TG neurons (De Regge et al., 2006a; De Regge et al., 2006b), we report that treatment of TG neurons with IFNα is sufficient to induce a quiescent HSV-1 and PRV infection *in vitro* that shows strong similarities to in vivo latency, thereby providing a novel and unique in vitro model to study HSV/PRV latency and reactivation and suggesting that IFNα may represent a key immune component involved in efficient establishment of alphaherpesvirus latency in sensory neurons.

## 2. Material and methods

### *2.1. Ethics statement*

Trigeminal ganglia were derived from animals that were euthanized at the Faculty of Veterinary Medicine, Ghent University, Belgium, according to FELASA guidelines (Federation of European Laboratory Animal Science Associations).

### *2.2. Cells and viruses*

Wild type PRV strain Becker (Card et al., 1990) was propagated on Swine Testicle cells. Wild type HSV-1 strain F (Ejercito et al., 1986) and HSV-1 mutants SΔUS5-LacZ (Balan et al., 1994) and LbetaA (Lachmann & Efstathiou, 1997) were propagated on Vero cells.

### *2.3. Cultivation and inoculation of primary trigeminal ganglion neuronal cultures in a two-chamber model*

Porcine trigeminal ganglia were excised from 2 to 4 week old piglets and dissociated by enzymatic digestion with 0.2% collagenase A (Roche)(17). The harvested cells were resuspended in culture medium (MEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mg/ml kanamycin and 30 ng/ml nerve growth factor (Sigma) and seeded in the inner chamber of an *in vitro* two-chamber model. The two-chamber model consists of a polystyrene cloning cylinder (Sigma) that is fixed with silicon grease on a collagen coated cover glass inserted in a 6 well plate (De Regge et al., 2006a). The inside of the cylinder forms the inner chamber, the outside forms the outer chamber. One day after seeding, cultures are washed with RPMI (Gibco) to remove non-adherent cells and from then on, culture medium is changed three times a week. After two to three weeks of cultivation, when clear axon growth can be observed in the outer chamber, two-chamber models are ready for inoculation with virus. Inoculation with all viruses used was done by adding $2x10^7$ PFU to the outer chamber. For PRV, two hours after inoculation of the outer chamber, medium containing PRV was removed and the outer chamber was washed twice with culture medium. Afterwards, new culture medium supplemented with polyclonal antibodies to PRV and guinea pig complement (Sigma) was added to prevent continuous infection pressure from the outer chamber to neurons in the inner chamber. For HSV-1, the virus was removed at 48h after inoculation by washing and new culture medium supplemented with monoclonal antibodies to HSV-1 gD and guinea pig complement was added.

### *2.4. Antibodies, cytokines and chemicals*

Polyclonal porcine FITC-labeled anti-PRV antibodies (Nauwynck & Pensaert, 1995) were used to detect late PRV proteins gB, gD and gE (Geenen et al., 2005). Monoclonal mouse-anti-PRV gD antibody 13D12 was described earlier (Nauwynck & Pensaert, 1995). Polyclonal rabbit-anti-PRV IE180 antibody was a kind gift from E. Tabarés (Universidad Autonoma de Madrid, Spain). Monoclonal mouse-anti-HSV-1 gD (124/468) and –anti-HSV-1 ICP4 (sc56986) were purchased from Santa Cruz Biotechnology and the neuronal marker rabbit-anti-neurofilament 200 from Sigma. Texas red-labeled goat-anti-rabbit antibodies and FITC-labeled goat-anti-mouse antibodies were from Invitrogen. Recombinant porcine IFNα and IFNγ were purchased from R&D and X-gal and forskolin were obtained from Sigma.

### *2.5. Quantification of the percentage viral antigen or β-galactosidase positive infected neurons*

The ratio between viral antigen positive or β-galactosidase positive neurons in the inner chamber to the number of axons in the outer chamber after different treatments was determined by calculating viral antigen positive neuronal cell bodies (immunofluorescence) or beta-galactosidase positive neurons (X-gal) in the inner chamber and immunofluorescently labeled axons in the outer chamber. For each experiment, at least 25 neurons with outgrowth to the outer chamber were examined. Data shown represent means ± s.e.m. of independent triplicate assays.

### *2.6. Immunofluorescence staining procedures*

Two-chamber systems to be used for immunofluorescent detection of viral antigen positive neurons were washed in PBS and fixed in 100% methanol for 20 min at -20°C. In two-chamber systems to be used for analysis of β-galactosidase positive neurons in the inner chamber, the outer chamber was fixed in 4% paraformaldehyde in PBS for 10 min and subsequently permeabilized in 0.2% TritonX-100 in PBS for 2 min. All antibodies were diluted in PBS, all to a dilution of 1:100. Cells were incubated with each antibody for 1h at 37°C and were washed two times between all incubation steps and after the last incubation step.

### *2.7. Detection of β-galactosidase activity*

Cells in the inner chamber of two-chamber systems were fixed in 2% paraformaldehyde-0.2% glutaraldehyde in PBS for 15 min at RT and subsequently incubated with staining buffer (0.01% Na-deoxycholate, 0.02% NP40, 2 mM $MgCl_2$, 4.5 mM potassium ferricyanide, 4.5 mM potassium ferrocyanide in PBS) for 5min at RT, followed by incubation

with X-gal buffer (staining buffer supplemented with 1 mg/ml X-gal) for 4h at 37°C. Afterwards, the inner chamber was washed twice with PBS and immediately analyzed by light microscopy.

### *2.8. RT-PCR*

RNA from cells grown in the inner chamber of two-chamber systems was isolated and purified using the Trizol Plus RNA purification kit (Invitrogen) according to manufacturer's instructions, followed by a DNaseI digestion to degrade any contaminating DNA. RNA was reverse transcribed using SuperScript III RT enzyme (Invitrogen) according to manufacturer's instructions. OligodT primers were used for reverse transcription of actin, PRV IE180, PRV gB, PRV LAT, HSV-1 ICP0, HSV-1 gB and HSV-1 gD RNA and a gene specific primer (GSP-LAT) that allows amplification of the 2kb LAT intron (Table 1) was used for reverse transcription of HSV-1 LAT RNA. The cDNA was then amplified by PCR using the AccuPrime Taq DNA polymerase system (Invitrogen). The sequences of primers, annealing temperatures and predicted lengths of amplified fragments can be found in Table 1. Amplified PCR fragments were analyzed by agarose gel electrophoresis and staining with Ethidium Bromide. The specificity of amplified fragments was verified by predicted sizes and by sequencing fragments that were purified from the agarose gel. Purified fragments were subjected to cycle sequencing with a Big Dye Terminator Cycle Sequencing kit v1.1 (Applied Biosystems) and cycle sequencing reaction products were purified using ethanol precipitation and separated on an ABI Genetic Analyzer 310 (Applied Biosystems). Obtained fragment sequences were compared with the NCBI nucleotide collection (nr/nt) database using MegaBlast.

**Table 1: RT-PCR specifications**

| primer | | | sequence (5'-3') | annealing temp. | predicted length |
|---|---|---|---|---|---|
| porcine | actin | forward: | ATGCAGAAGGAGATCACGGC | 50 | 199 |
| | | reverse: | AGTCCGCCTAGAAGCATTTG | | |
| PRV | IE180 | forward: | ACGCGAGAGGAAGTAGGGAG | 57 | 393 |
| | | reverse: | GTACCTGCACCGCAGTGAAG | | |
| | gB | forward: | CCTCCTCGACGATGCAGTTG | 59 | 281 |
| | | reverse: | CACGAACCGCTTCACAGACC | | |
| | LAT | forward: | CATAAAGCCAGTTGAAGACGGGG | 59 | 526 |
| | | reverse: | TAGAGGGTCTTGGGGATGTTGG | | |
| HSV-1 | ICP0 | forward: | GCCCACTATCAGGTACAC | 55 | 301 |
| | | reverse: | CACGGAACTGTTCGAGAC | | |
| | gB | forward: | TGGCGTCGGAAGAGAATCGG | 59 | 213 |
| | | reverse: | AGCAGGTCGACGGCTTCTAC | | |
| | gD | forward: | AGCCAAGGGCTCCTGTAAG | 58 | 352 |
| | | reverse: | GTCCTGGATCGACGGTATGTG | | |
| | GSP-LAT | | TGGTGGACCAGACGGGAAAC | | |
| | LAT | forward: | CCGCGATACATCCAACAC | 53 | 383 |
| | | reverse: | GAACAGCCTCTGGATGAC | | |

Primer sequences and annealing temperatures (°C) used in RT-PCR and predicted length (bp) of amplified fragments.

## *2.9. Confocal microscopy*

Samples were analyzed on a Leica TCS SP2 laser scanning spectrum confocal system (Leica Microsystems GmbH) linked to a Leica DM IRBE microscope. Images were taken using a 63x oil objective (NA 1.40-0.60) at RT and using Leica confocal acquisition software. Adjustments of brightness and contrast were applied to the entire images and were done using Adobe Photoshop (Adobe Systems Inc.).

## 3. Results

### *3.1. PRV and HSV-1 proceed to productive replication in porcine TG neurons*

Porcine TG neurons grown in two-chamber models were infected with PRV or HSV-1 by addition of virus to the outer chamber. For PRV, at 24hpi, the vast majority of neurons (98%) that had axons growing in the outer chamber were positive for late viral antigens (Fig. 1C), indicating that PRV proceeds to productive infection in virtually all infected neurons. In some infected neurons at 24hpi, late PRV protein expression was limited to the neuronal cell body (Fig. 1A) while in others, infection had already spread to non-neuronal cells surrounding the cell body (Fig. 1B). Initiation of productive infection was less efficient when TG neurons were infected with HSV-1, but still resulted in 12% of the neurons with axonal outgrowth in the outer chamber being positive for the late HSV-1 antigen gD at 48hpi (Fig. 1F). As for PRV, some neurons showed HSV-1 gD expression limited to the neuronal cell body (Fig. 1D) while in others infection had already spread from the cell body to surrounding non-neuronal cells at this time point (Fig. 1E). To ensure that the substantial difference in percentage of productively infected neurons between HSV-1 and PRV was not due to the difference in antibodies used (polyclonal mixture for PRV versus monoclonal gD-specific for HSV-1), experiments with PRV were repeated using a PRV gD-specific monoclonal antibody. Again, the vast majority (75.3 ± 6.2%) of neurons with axonal outgrowth in the outer chamber were positive at 24hpi with PRV. To analyze whether the limited percentage of productively HSV-1 infected neurons is due to a hampered HSV-1 entry in porcine TG neurons, neurons were infected with the SΔUS5-LacZ HSV-1 mutant carrying the reporter gene LacZ under control of the human CMV MIEP promoter inserted in the non-essential US5 gene (15). X-gal staining of infected cultures showed that over 90% of neurons with axons growing into the outer chamber were beta-galactosidase positive at 24hpi (Fig. 1F), indicating that HSV-1 efficiently enters porcine TG neurons but does not efficiently initiate productive replication. To evaluate whether the block in productive HSV-1 replication occurs before or after expression of the earliest viral proteins (immediate-early or IE proteins), HSV-1 experiments were repeated using an ICP4-specific monoclonal antibody. This resulted in a percentage of positive neurons (11.7 ± 1.5%) similar to the percentage observed using gD-specific antibodies, indicating that the neurons that do not proceed to productive HSV-1 replication are halted before detectable ICP4 protein expression. Overall, these results indicate that upon viral entry, PRV efficiently proceeds to productive replication. For HSV-1, ± 12% of infected neurons proceed to productive replication whereas the others are halted at a stage very early in infection.

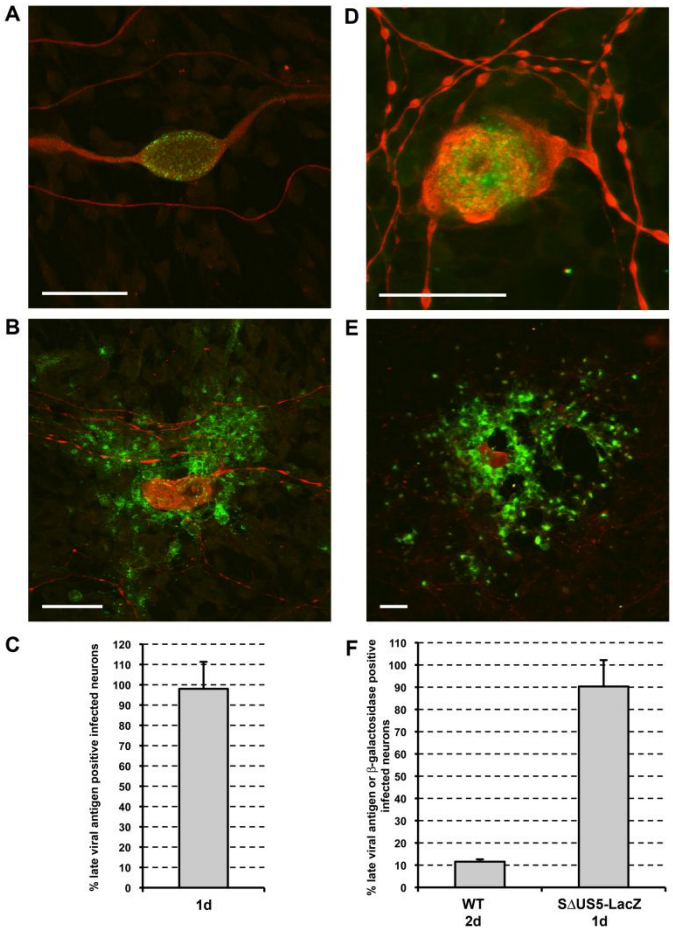


**Figure 1. Productive replication of PRV and HSV-1 in porcine TG neurons.** Confocal images of TG neuronal cultures in the inner chamber at 24hpi with PRV (A,B) and 48hpi with HSV-1 (D,E) stained for neurofilament (red) and late viral antigens (green) (bar = 50 µm). Percentage of neurons with axons growing out to the outer chamber that show viral antigens at 24hpi with PRV (C) and 48hpi with wt HSV-1 (F, left bar) and β-galactosidase activity at 24hpi with SΔUS5-LacZ HSV-1 (F, right bar). Data represent the mean ± s.e.m. of three independent experiments.

## *3.2. Interferon alpha suppresses PRV and HSV-1 productive replication in TG neurons for several days*

The effect of IFNα on the expression of late viral proteins upon inoculation with both viruses was analyzed. Two-chamber systems were pretreated with IFNα for 24h and after infection, IFNα remained present in the inner chamber for the entire experiment. A dose dependent decrease was observed in the number of PRV infected neurons expressing late viral proteins at 24hpi, ranging from 36 ± 1% late viral antigen positive neurons with 0.5 U/ml IFNα (data not shown) to 2 ± 1% with 500 U/ml (Fig. 2A). The latter concentration was selected for all further experiments. The suppressive effect of IFNα on PRV replication was sustained over a longer period of time since at 5dpi, still only 10 ± 4% of infected neurons were late viral antigen positive (Fig. 2A). For HSV-1, the suppressive effect of IFNα was even more pronounced since not a single HSV-1 gD positive infected neuron was detected, both at 2 and 5dpi (Fig. 2B). Both for PRV and HSV-1, in the absence of IFNα, cytopathic effect was complete well before 5dpi. These results indicate that IFNα is able to efficiently suppress alphaherpesvirus replication in porcine TG neurons for several days.

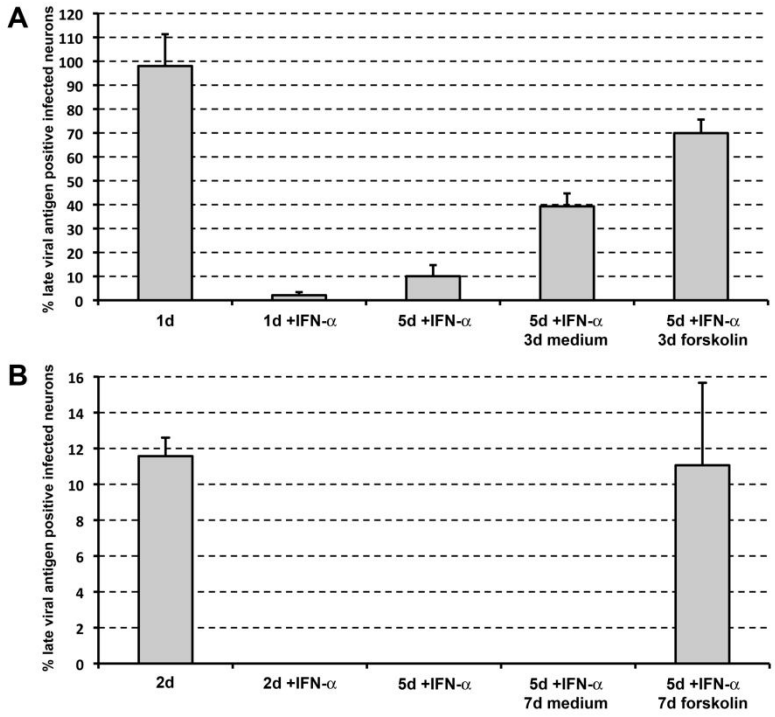


**Figure 2. IFNα induces a reactivatable, latent PRV and HSV-1 infection in porcine TG neurons.** Percentage infected neurons that are late viral antigen positive at 1, 5 and 8dpi with PRV (A) and at 2, 5 and 12dpi with HSV-1 (B) in the presence or absence of 500 U/ml IFNα. For the neurons fixed at 8dpi with PRV and 12dpi with HSV-1, medium containing IFNα was washed out at 5dpi and replaced with new culture medium or new culture medium supplemented with forskolin (200 µM). Data represent the mean ± s.e.m. of three independent experiments.

## *3.3. Interferon alpha suppresses PRV IE180 and HSV-1 ICP4 protein expression in TG neurons*

To determine whether IFNα not only suppresses late viral protein expression, but may also affect IE protein expression, levels of ICP4 of HSV-1 or the corresponding IE180 protein of PRV were analyzed in IFNα–treated two-chamber systems. For HSV-1, IFNα treatment resulted in fast and efficient suppression of ICP4 protein levels, leading to undetectable ICP4 levels at both 2dpi and 5dpi. For PRV, IFNα-mediated suppression of IE180 appeared to be less efficient and slower, resulting in 91.5 ± 14.8% of IE180-positive neurons with axonal outgrowth in the outer chamber at 1dpi, and 46.1 ± 3.2% of IE180-positive neurons at 5dpi. These results indicate that IFNα is able to suppress alphaherpesvirus IE protein levels in porcine TG neurons, and that this appears to occur more efficiently in HSV-1-infected neurons compared to PRV-infected neurons.

### *3.4. Interferon alpha induces a stably suppressed quiescent PRV and HSV-1 infection*

During virus latency, the virus is present in a stably suppressed state. In other words, latency persists, even when the suppressive agent is removed. To analyze whether IFNα is able to induce such a stably suppressed state of alphaherpesvirus infection, we analyzed whether or not withdrawal of IFNα at 5dpi resulted in re-expression of late viral antigens in TG neurons. For PRV, infection was stably suppressed in 60% of the neurons, as they did not express detectable levels of late viral proteins at 3 days post IFNα withdrawal (Fig. 2A). For HSV-1, none of the neurons initiated expression of detectable levels of HSV-1 gD even at 7 days after withdrawal of IFNα (12dpi) (Fig. 2B), showing that all HSV-1 infected TG neurons were in a stably suppressed quiescent state of infection.

### *3.5. Forskolin treatment triggers PRV and HSV-1 reactivation in neurons containing quiescent virus*

Alphaherpesvirus latency is defined as a functional viral genome retained in neurons in the absence of virus particles but capable to reactivate resulting in production of new infectious virus (Decman et al., 2005). Forskolin is a known stimulus of alphaherpesvirus reactivation (Colgin et al., 2001; Danaher et al., 2003; Smith et al., 1992). Therefore, we analyzed whether forskolin was able to reactivate PRV and HSV-1 in the neurons containing stably suppressed virus. Again, IFNα was withdrawn at 5dpi and medium supplemented with forskolin (200 μM) was added. For PRV, 70% of the infected neurons were late viral antigen positive at 3 days post IFNα withdrawal (Fig. 2A), often with virus spread to neighboring non-neuronal cells, indicating that forskolin treatment induced reactivation of PRV in 50% of neurons that contained stably suppressed virus at 5dpi. For HSV-1, a similar experiment was performed but medium supplemented with forskolin was added twice, at 5dpi (when IFNα was withdrawn) and again at 8dpi. Analysis of neurons at 12dpi showed that forskolin had induced reactivation of HSV-1 since 11% of infected neurons were positive for HSV-1 gD at that time point (compared to 0% without forskolin)(Fig. 2B), often with spread of the virus to neighboring non-neuronal cells. Reactivation ultimately led to complete cytopathic effect in the inner chamber (data not shown). Forskolin triggers reactivation but was found not to prevent the IFNα–mediated suppression of productive viral replication, since addition of IFNα and forskolin in parallel did not influence the ability of IFNα to suppress productive viral replication (data not shown). Overall, these data show that forskolin can reactivate HSV-1 and PRV from IFNα–induced quiescence.

## *3.6. PRV and HSV-1 express LATs during in vitro latency-like quiescence*

During HSV-1 and PRV latency *in vivo*, expression of LATs is frequently observed (Cheung, 1989; Stevens et al., 1987). To determine LAT expression during *in vitro* latency-like quiescence by wild type HSV-1 and PRV, RT-PCR was performed on RNA isolated from TG neurons derived from these two-chamber models that were i) mock infected, ii) productively infected, and iii) uniformly quiescently infected (Fig. 3A,B). For PRV, all three quiescently infected cultures (CPE negative at 5dpi) examined were negative for immediate-early (IE180) and late (gB) viral RNA but one was positive for LAT RNA (Fig. 3A). For HSV-1, all three quiescently infected cultures examined were negative for late (gB and gD) viral RNA but 2 were positive for LATs (Fig. 3B). RT-PCR assays contained quite prominent aspecific amplification signals, which are likely due to the high number of PCR cycles needed to detect sufficient signal. Specificity of the bands was confirmed via sequencing and contamination by viral genomic DNA was excluded by DNAseI treatment and by performing control reactions in the absence of reverse transcriptase. The RT-PCR assays show that both PRV and HSV-1 express detectable levels of LATs in a subset of infected porcine TG neurons during IFNα induced latency-like quiescence. Interestingly, one of the three quiescently HSV-1 infected cultures examined, which were all negative for gD RNA, was positive for ICP0 transcripts (Fig. 3B).

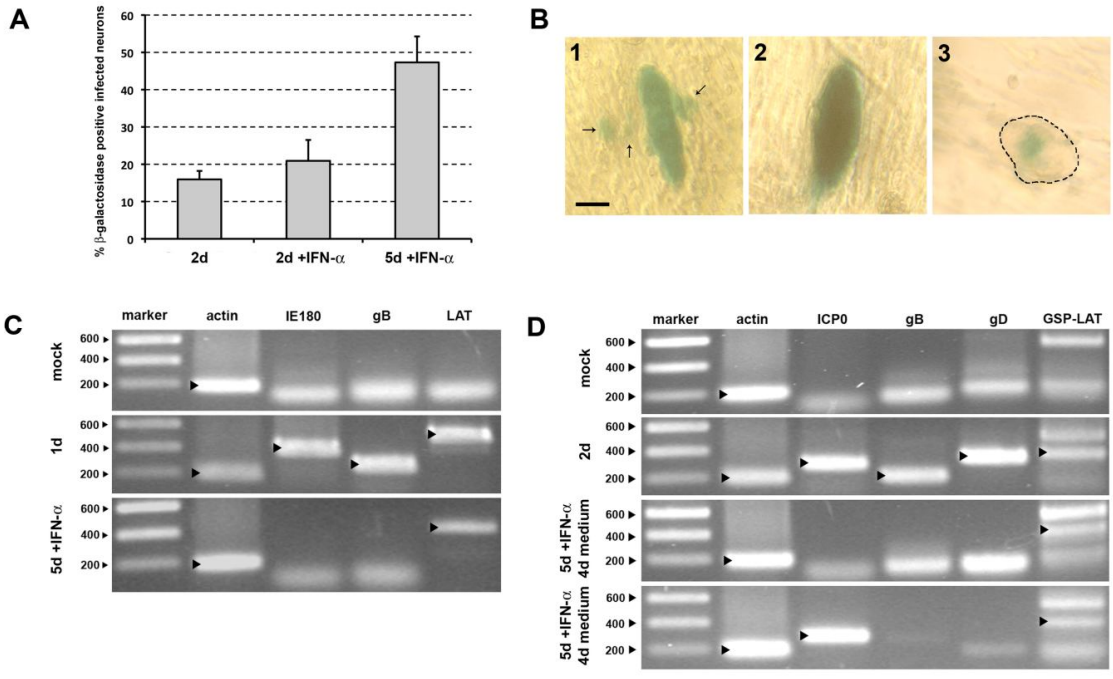


**Figure 3. PRV and HSV-1 express LATs during *in vitro* latency.** (A) Percentage of infected neurons positive for LAT promoter-driven β-galactosidase at 2 and 5dpi with HSV-1 LbetaA in the presence or absence of 500 U/ml IFNα. Data represent the mean ± s.e.m. of three independent experiments. (B) Light microscopic images of uniform (1,2) and focal (3) LAT promoter-driven β-galactosidase distribution during the acute stage (2dpi without IFNα, 1, 2) or the latent stage (5dpi with IFNα, 3) of infection with HSV-1 LbetaA. Arrows point to infected non-

neuronal cells (1), dashed line marks contour of neuronal cell body in (3) (bar = 20 µm). (C,D) RT-PCR analysis of actin and viral immediate-early (IE180 and ICP0), late (gB and gD) and LAT transcript RNA isolated from neuronal cultures that were either mock infected, productively infected with PRV (C, 1dpi) or HSV-1 (D, 2dpi), or latently infected with PRV (C, 5dpi with IFNα) or HSV-1 (D, 9dpi, 4 days post IFNα withdrawal). For each condition three different samples were analyzed and representative gels are shown. For HSV-1, two samples of 9dpi, 4 days post IFNα withdrawal are shown, one without and one with detectable ICP0 transcript expression. Specific bands are marked with a black arrowhead.

## *3.7. The HSV-1 LAT promoter is activated over time during latency-like in vitro quiescence*

For HSV-1, LAT promoter activity was assessed during IFNα induced latency-like quiescence *in vitro*. Two-chamber systems were infected with the LbetaA HSV-1 mutant carrying a LacZ reporter gene under control of the LAT promoter (Lachmann & Efstathiou, 1997) in the presence or absence of IFNα. X-gal staining of cultures not treated with IFNα showed that 16% of infected neurons were β-galactosidase positive at 48hpi (Fig. 3A). This percentage is similar to the percentage of HSV-1 gD positive neurons after wild type HSV-1 infection at 48hpi (12%). In the presence of IFNα, we observed an increase of β-galactosidase positive infected neurons over time to about 50% positive neurons at 5dpi (Fig. 3A). As a control, no evidence for β-galactosidase activity was found at 5dpi in the presence of IFNα in neurons infected with the SΔUS5-LacZ HSV-1 mutant carrying the reporter gene LacZ under control of the human CMV MIEP promoter. The activation of the LAT promoter over time suggests a gradual de-repression of the LAT promoter. Analysis of β-galactosidase distribution in ± 20 neurons in either lytically infected neurons (2dpi in the absence of IFNα) or quiescently infected neurons (5dpi in the presence of IFNα) indicated that in lytically infected neurons, β-galactosidase distribution was generally uniform throughout the neuronal cell body (Fig. 3B 1, 2) and sometimes associated with spread of infection to surrounding non-neuronal cells (Fig. 3B 1), while a more focal β-galactosidase distribution was frequently found in quiescently infected neurons (Fig. 3B 3). In summary, in the presence of IFNα, HSV-1 LAT promotor activity can be observed in up to ± 50% of infected neurons at 5dpi. Since this percentage is higher than the percentage of neurons that proceed to a productive replication in the absence of interferon (12%, Fig1F), this suggests that at least a fraction of the neurons that do not show the capacity to proceed to productive replication in the absence of IFNα do show LAT promoter activity during prolonged incubation with IFNα.

## 4. Discussion

In the current report, we show that addition of IFNα to two-chamber systems of porcine TG neurons is sufficient to induce a quiescent PRV and HSV-1 infection that shows strong similarities to *in vivo* latency.

Besides the insights in alphaherpevirus latency/reactivation generated by in vivo studies in animals, ex vivo explants of latently infected neurons, and from biopsies of deceased individuals, over the years, several very valuable in vitro models have been developed that have increased our understanding of HSV quiescence and latency. Some of the most notable of these in vitro systems are based on the use of antiviral drugs, mainly nucleoside analogues that act as viral DNA chain terminators. Such drugs have been shown to induce a stably suppressed state of HSV infection that shows similarities to *in vivo* latency. Cells used in these models vary from fibroblasts over neuronal cell lines (e.g. PC12) to primary rat dorsal root ganglion neurons (Danaher et al., 1999; Wigdahl et al., 1982; Wigdahl et al., 1984a; Wigdahl et al., 1984b; Wilcox et al., 1997). Like noncytotoxic lytic granules and type II interferon, antiviral drugs like acyclovir have also been shown to have the capacity to prevent HSV reactivation in vitro upon explantation of TG neurons obtained from latently infected mice (Halford et al., 2001; Knickelbein et al., 2008; Liu et al., 2001). Some of the other *in vitro* models of HSV latency are based on the use of replication-defective, attenuated virus strains (Wilcox et al., 1992). Still other models are based on a reversible, temperature-dependent suppression of virus replication (Kondo et al., 1990; Wrzos & Rapp, 1987). Some previous in vitro latency models contained IFN – however, simultaneous addition of nucleoside analogues was required to establish quiescence (Wigdahl et al., 1982; Wigdahl et al., 1984a; Wigdahl et al., 1984b). Since addition of nucleoside analogues without IFN also prevents viral replication and leads to quiescence (Wilcox et al., 1997), these data were inconclusive on a potential involvement of IFN in latency establishment. In our current model, addition of IFNα is sufficient to establish latency-like quiescence, without the need for nucleoside analogues. This important difference is perhaps due to the use of a two-chamber system that allows an in vivo-like route of infection of neurons, via retrograde axonal spread. It has been suggested before that the long distance retrograde transport of HSV in neurons results in reduced levels of the viral VP16 transactivator reaching the nucleus (Roizman & Sears, 1987). Without a two-chamber system, virus can access the neurons via the cell body, thereby circumventing retrograde axonal spread.

The obtained results indicate a strong similarity between the currently described *in vitro* quiescence and alphaherpesvirus latency *in vivo*. First of all, our results show that both PRV and HSV-1 express detectable levels of LATs in a subset of quiescently infected *in vitro*

systems. It will be interesting to further explore LAT expression in the *in vitro* latency model, e.g. by determining nuclear localization of the LAT transcripts. Our observation that not all cultures contain detectable LAT expression appears to be in line with the notion that *in vivo* a varying number of latently HSV-1 infected human TG neurons expresses detectable levels of LATs (Wang et al., 2005). We observed that some quiescently infected cultures are positive for both LAT and ICP0 transcripts. Although we cannot formally rule out the possibility that these LAT/ICP0 mRNA double-positive quiescent cultures may consist of neurons that express either LAT or ICP0 mRNA, it is also possible that quiescently infected neurons express both transcripts at the same time. Interestingly, there is increasing evidence for the presence of ICP0 transcripts in ganglia of humans and mice latently infected with HSV-1 (Chen et al., 2002; Derfuss et al., 2009; Maillet et al., 2006), and our current data may therefore be in line with indications that ICP0 protein expression in latently infected neurons may at least partly be blocked at the post-transcriptional level, probably due to microRNA activity of LATs and perhaps other, unknown, viral and host factors (Chen et al., 2002; Maillet et al., 2006; Thompson et al., 2003; Umbach et al., 2008). Using a previously described HSV-1 recombinant expressing β-galactosidase under control of the LAT promoter (Lachmann & Efstathiou, 1997), a focal β-galactosidase distribution was observed in latently infected neurons. Such a focal β-galactosidase distribution was also observed during *in vivo* latency in ganglia of mice latently infected with the LbetaA mutant and with another HSV mutant expressing LacZ under control of the LAT promoter (Lachmann & Efstathiou, 1997; Ho & Mocrski, 1989). It has been suggested that the focal β-galactosidase distribution may be due to physiological differences between lytically and latently infected neurons.

Our results indicate that IFNα leads to efficient establishment of PRV and HSV-1 latency-like quiescence in TG neurons *in vitro*. Based on these data, it is tempting to speculate that IFNα also represents a key immune component involved in the efficient establishment of alphaherpesvirus latency in sensory neurons *in vivo*. Some in vivo data may support this hypothesis: several reports indicate that impaired IFN responses *in vivo* can result in uncontrolled lytic virus replication and, often fatal, afflictions of the central nervous system, including herpes simplex encephalitis in humans (Casrouge et al., 2006; Zhang et al., 2007), and increased replication efficiency of strongly attenuated HSV-1 mutants in TG neurons of mice (Leib et al., 1999). Based on the current *in vitro* data, it will be interesting to further investigate if type I IFNs indeed act as a double-edged sword *in vivo*: on the one hand protecting the host from severe infection by alphaherpesviruses, on the other hand contributing to their lifelong persistence in a latent form.

*In vivo*, there is evidence that type II IFN (IFNγ), mainly produced by $CD8^+$ T lymphocytes, plays only a minor additional role to type I IFNs in controlling early acute

infection (Leib et al., 1999; Cantin et al., 1995) but is important to prevent reactivation of alphaherpesviruses from latency (Liu et al., 2001; Khanna et al., 2004). Further in line with the similarity between alphaherpesvirus *in vivo* latency-like quiescence and the currently described *in vitro* model, we found that IFNγ, although unable to suppress PRV replication over longer periods of time and therefore unable to induce PRV quiescence *in vitro* as observed for IFNα, is able to maintain PRV quiescence *in vitro* (data not shown). Although speculative at this point, together with the literature data, this may suggest that especially type I IFNs, as a crucial factor of the innate antiviral immunity, may be of importance during establishment of latency whereas type II IFN produced by the adaptive immunity are of crucial importance in maintaining the virus in a latent state and preventing reactivation. It will be interesting to determine whether the latency-like quiescent state of infection is the result of IFNα directly promoting the establishment of latency/quiescence, or, alternatively, of an IFNα-mediated inhibition of lytic replication that indirectly promotes latency/quiescence. Dissecting which IFNα-induced effectors are involved in the induction of quiescence will aid to clarify this. Both in PRV and HSV, IFNα suppressed protein expression levels of IE genes ICP4/IE180, suggesting that IFNα–mediated IE suppression may be a key element in the ability of this innate immune effector to establish quiescence. In line with this hypothesis, we observed that the percentage of neurons that still show detectable IE180 protein levels at 5dpi with PRV in the presence of IFNα correlates well with the percentage of neurons that are not in a stable quiescent infection at that time point and proceed to productive replication upon IFNα withdrawal (46% versus 40%).

In summary, this study presents a novel and unique *in vitro* system to dissect aspects of the latency/reactivation cycle of wild type alphaherpesviruses and points to IFNα as a potential driving force in efficient alphaherpesvirus latency establishement. In addition, the currently described in vitro model may provide a unique tool to screen possible drug candidates that interfere with the latency/reactivation cycle.

## 5. Acknowledgements

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# Chapter 4: Effects of interferon on immediate-early mRNA and protein levels in sensory neuronal cells infected with herpes simplex virus type 1 or pseudorabies virus

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## Abstract

Most alphaherpesviruses are able to establish latency in sensory neurons and reactivate upon specific stimuli to cause recurrent symptoms. We have previously shown that interferon (IFN) is capable of inducing a quiescent HSV-1 and PRV infection that strongly resembles *in vivo* latency in primary cultures of TG neurons. This IFN-induced latency-like quiescence was found to correlate with suppression of the immediate-early protein ICP4 in HSV-1 and its ortholog IE180 in PRV. Here, we mechanistically investigated the IFN-mediated suppression of ICP4 and IE180 in sensory neuronal cells. Using RT-qPCR, mRNA levels of either HSV ICP4 or PRV IE180 at 4hpi were mildly but not significantly different in IFN-treated samples versus control samples, whereas a strong reduction was observed at 8hpi and 12hpi. However, at 4hpi, HSV ICP4 but not PRV IE180 protein expression was already strongly reduced in IFN-treated samples. In line with this difference in IFN-mediated suppression of HSV ICP4 versus PRV IE180 protein levels, we found that IFN resulted in an increase in phosphorylation of the translation initiation factor eIF2α in HSV-infected but not in PRV-infected cells. The latter finding indicates that PRV efficiently circumvents IFN-mediated translation inhibition by interfering with phosphorylation of eIF2α.

## 1. Introduction

The human herpes simplex virus type 1 (HSV-1) and the porcine pseudorabies virus (PRV) are closely related and belong to the alphaherpesvirus subfamily of the Herpesviridae.

A central characteristic of most alphaherpesviruses is their ability to establish a lifelong latent infection in sensory neurons. During HSV-1 and PRV latency, no infectious virus or viral proteins are produced (Roizman and Knipe, 2001). Specific stimuli, often associated with stress and/or immune suppression, can lead to reactivation of the virus from latency, which may be associated with recurrent virus spread and disease symptoms, such as cold sores with HSV-1 (Sainz et al., 2001). The establishment and control of latency and the induction of reactivation are regulated by an incompletely understood interplay between the virus, the neuron and several components of the immune system (Decman et al., 2005).

The interferon (IFN)-mediated innate immunity is at the front line of host defence against viral infections. IFNs are able to interfere with several steps of virus replication. One important example is the IFN-mediated shutdown of mRNA translation through phosphorylation and thereby inactivation of the translation initiation factor eIF2α and through activation of RNase L (Goodbourn et al., 2000). IFNs have been reported to be very important in limiting replication and spread of alphaherpesviruses (Hendricks et al., 1991; Mikloska and Cunningham, 2001; Sainz and Halford, 2002). Recently, we have demonstrated that IFN is able to drive both HSV-1 and PRV into a latency-like quiescent state in in vitro cultures of sensory neurons (De Regge et al., 2010).

Importantly, IFN-mediated suppression of the immediate-early (IE) protein ICP4 of HSV-1 or its counterpart IE180 in PRV appeared to be a key step in the establishment of in vitro latency and IFN-mediated IE protein suppression was more rapid and efficient in HSV-1 compared to PRV (De Regge et al., 2010).

The aim of the current study was to further mechanistically investigate the suppressive effect of IFN on ICP4 and IE180 levels in sensory neuronal cells. We report that, at the mRNA level, IFN reduced ICP4 and IE180 mRNA expression to a similar extent, in both cases predominantly late in infection. At the protein level, IFNα was found to rapidly and efficiently shut down ICP4 protein expression whereas IE180 protein expression was diminished slower and less efficient, in line with our earlier observations. Remarkably, in HSV-1 infected sensory neuronal cells, IFN caused a rapid and marked phosphorylation and therefore inactivation of the translation initiation factor eIF2α, whereas no such phosphorylation could be observed in PRV-infected sensory neurons. The latter finding may explain the difference in efficiency of IFN-mediated IE suppression in HSV-1 versus PRV,

since it suggests that PRV may efficiently circumvent the IFN-mediated phosphorylation of eIF2α that normally would inhibit IE180 translation.

## 2. Materials and Methods

### *2.1. Cells and viruses*

Sensory neuronal cells originating from rat dorsal root ganglion neurons (50B11) cells were a kind gift from Dr. Höke (Department of Neurology, Johns Hopkins University). The cells were grown in neurobasal medium supplemented with 1,1% glucose (20%), 0,27% L-glutamine, 10% fetal calf serum, 2% B-27 and 0,1% blasticidin (Chen et al., 2007). Differentiation of the cells was obtained by treatment with forskolin (50μM) (Sigma) for 24h. HSV-1 strain VR-733 (Ejercito et al., 1968) was grown and titrated on Vero cells and stored at -80°C. PRV strain Becker (Card et al., 1990) was grown and titrated on Swine Testicle (ST) cells and stored at -80°C.

### *2.2. Interferons and antibodies*

Cells were pre-treated with a combination of 1000U/ml Universal Type I IFN-α (R&D) and 100ng/ml Recombinant Rat IFN-γ (R&D) for 24h before virus inoculation. IFN remained present during infection. HSV-1 mouse monoclonal anti-ICP4 (sc56986) was purchased from Santa Cruz. The rabbit polyclonal anti-IE180 antibody was a kind gift from E. Tabarés (Universidad Autonoma de Madrid, Spain). Rabbit monoclonal anti-actin (A2066) was purchased from Sigma-Aldrich. Mouse monoclonal anti-eIF2α (L57A5) and rabbit monoclonal anti-phospho-eIF2α (D9G8) were purchased from Cell Signalling. HRP-conjugated secondary goat anti-rabbit was purchased from Cell Signaling and goat anti-mouse antibodies were purchased from Dako Cytomation. Thapsigarin was purchased from Invitrogen.

### *2.3. Total RNA isolation and quantitative RT-PCR*

RNA from cultured cells was isolated using the Trizol Plus RNA purification kit from Invitrogen according to the manufacturer's protocol. Samples were treated with DNase I and subsequently RNA was converted into cDNA using the SuperScript III enzyme and oligodT primers (Invitrogen) according to the manufacturer's protocol. Subsequently quantitative PCR was performed using the SYBR Green dye from the SYBR Fast Bio-rad Readymix Kit (Sopachem). The primers that were used are 5' CGACACGGATCCACGACCC 3' and 5'GATCCCCCTCCCGCGCTTCGTCCG 3' at 60°C for ICP4 (Kramer and Coen, 1995), 5' ACGCGAGAGGAAGTAGGGAG 3' and 5'GTACCTGCACCGCAGTGAAG 3' at 57°C for IE180, 5'CTGCCGTCTGGAGAAACCTG 3' and 5' CCACCACCCTGTTGCTGTAG 3' at 53°C

for the reference gene GAPDH. GAPDH expression was not affected by IFN treatment or infection. RNA samples were prepared as four independent replicates and results of each replicate were normalized to GAPDH.

### *2.4. Western blotting*

SDS/PAGE and Western blotting were performed as described previously (Deruelle et al., 2007). Blots were blocked in 5% non-fat dry milk in PBS/Tween-20 for 1h at room temperature. The blots were incubated for 1h or overnight (according to the manufacturer's instructions) with primary antibodies and washed 3 times in 0.1% TBS/Tween-20 (TBS-T). Blots were incubated with HRP-conjugated secondary antibodies for 1h at room temperature and after several washing steps they were developed with enhanced chemiluminescence (ECL Plus; GE Healthcare).

### *2.5. Immunofluorescence staining*

Cells were washed in PBS and fixed at room temperature with 3% paraformaldehyde in PBS for 10min, washed in PBS and permeabilized at room temperature in 0,2% Triton-X-100 in PBS for 2min. After washing in PBS, cells were incubated for 1h at 37°C with P-eIF2α primary antibody (1/100) diluted in PBS, washed twice in PBS and subsequently incubated for 1h at 37°C with the goat-anti-rabbit FITC secondary antibody (Invitrogen) (1/200) diluted in PBS. Afterwards cells were washed in PBS and incubated at room temperature for 10min with Hoechst 33342 (Invitrogen) (1/200) diluted in PBS. Then cells were analyzed using the C2 confocal microscope from Nikon.

### *2.6 Statistics*

RNA samples were prepared as four independent replicates and results of each replicate were normalized to GAPDH. Means and standard deviations were calculated and subsequently the ratio of IFN-treated versus untreated values was determined. Statistical analysis was performed by using Graphpad Prism software using a t-test ($p < 0.05$).

# 3. Results

## *3.1. mRNA levels of PRV IE180 and HSV-1 ICP4 are suppressed with similar and relatively late kinetics*

Earlier, we reported that IFN-mediated suppression of ICP4 in HSV-1 and its counterpart IE180 in PRV correlates with establishment of in vitro latency in primary sensory neurons and that this occurs more rapid and efficient after infection with HSV-1 than with PRV (De Regge et al., 2010). To determine whether IFN-mediated IE suppression is noticeable at the mRNA level, RT-qPCR assays were performed. 50B11 sensory neuronal cells were either or not pre-treated with IFN for 24h and subsequently infected with PRV Becker or HSV-1 VR-733 (MOI 1). Total RNA was isolated at 4hpi, 8hpi or 12hpi and converted to cDNA followed by quantitative PCR. The results were normalized to GAPDH and the ratio of IE mRNA levels in IFN-treated versus untreated cell samples was calculated. Figure 1 shows that both IE180 and ICP4 mRNA levels were mildly and non-significantly reduced at 4hpi. At the later time points (8hpi and 12hpi), however, we did see a very strong and significant reduction in both IE180 and ICP4 mRNA expression. These data suggest that IE transcription is not substantially affected by IFN early in infection, allowing leaky transcription of both ICP4 and IE180 early in infection. Later in infection (>4hpi), a substantial suppression of IE mRNA, suggestive for a block in transcription, was observed for both HSV-1 and PRV. No apparent differences could be observed between HSV-1 and PRV.

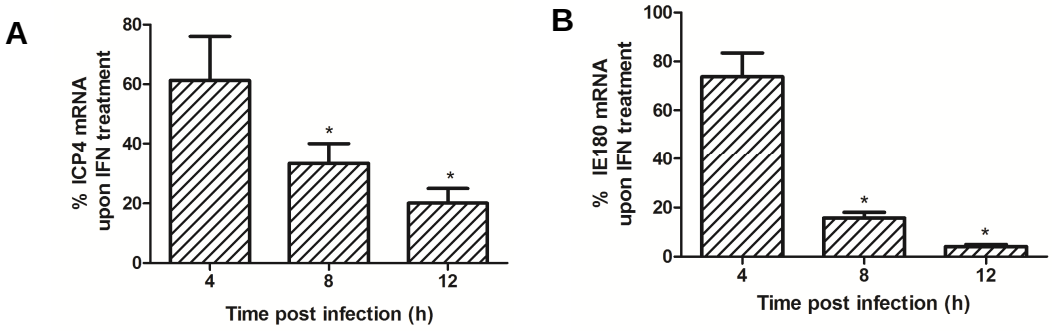


**Figure 1: IFN suppresses mRNA levels of PRV IE180 and HSV-1 ICP4 with similar kinetics.** 50B11 cells were pre-treated or not with IFN for 24h before virus inoculation. Total RNA samples were isolated at 4hpi, 8hpi or 12hpi with (A) PRV Becker or (B) HSV-1 VR-733 (MOI 1). RNA was converted to cDNA followed by RT-qPCR for GADPH, IE180 (PRV) and ICP4 (HSV-1)**.** Viral mRNAs were normalized to GAPDH and the fold change was determined by calculating the ratio between IFN-treated and untreated samples. * $p < 0.05$

### *3.2. Protein levels of PRV IE180 are suppressed later compared to HSV-1 ICP4*

To determine whether the relatively slow IFN-mediated suppression of ICP4 and IE180 mRNA levels correlate with the kinetics of IE protein levels in sensory neuronal cells, Western blot assays were performed. 50B11 cells were either or not pre-treated with IFN for 24h and subsequently infected with PRV Becker or HSV-1 VR-733 (MOI 1). Cell lysates were collected at 4h, 8h and 12hpi and immunoblotting was performed for IE180 and actin (loading control) for PRV samples and ICP4 and actin (loading control) for HSV-1 samples. For HSV-1, IFN treatment resulted in a rapid and strong reduction of ICP4 protein expression at 4hpi (Fig. 2). For PRV, however, IFN-mediated IE180 protein suppression was markedly slower. IE180 protein levels were not noticeably suppressed at 4hpi and were only substantially reduced later in infection (8hpi and 12hpi) (Fig. 2). These results implicate a rapid translational inhibition of HSV-1 ICP4 compared to a markedly slower suppression of PRV IE180 protein levels.

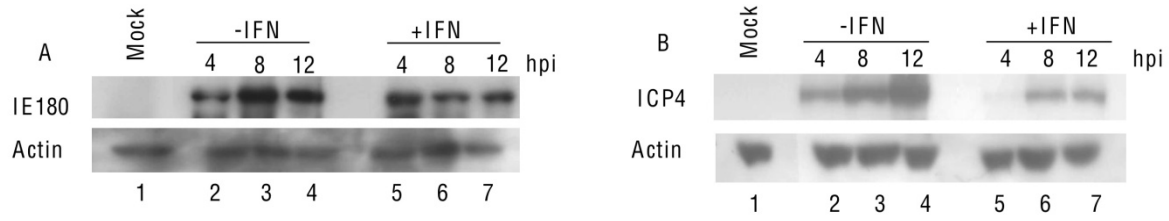


**Figure 2 : Protein levels of PRV IE180 are suppressed later compared to HSV-1 ICP4 upon IFN treatment.** 50B11 cells were either or not pre-treated with IFN for 24h before virus inoculation. Cell lysates were collected at 4hpi, 8hpi or 12hpi with (A) PRV Becker or (B) HSV-1 VR-733 (MOI 1). Western blotting was performed for actin, IE180 (PRV) and ICP4 (HSV-1).

### *3.3. PRV strongly suppresses IFN-induced phosphorylation of eIF2α*

Our results suggest that IFN-mediated translational shutdown in 50B11 cells is markedly more rapid and efficient for HSV-1 ICP4 compared to PRV IE180. Through upregulation of protein kinase R (PKR), IFN induces phosphorylation of the translation initiation factor eIF2α, which shuts down translation. To determine whether the observed differences in IFN-mediated HSV-1 ICP4 versus PRV IE180 protein suppression in 50B11 cells correlate with differences in IFN-mediated phosphorylation of the translation initiation factor eIF2α, immunoblotting was performed. Thapsigargin, an inhibitor of sarco/ER Ca2+ ATPases that induces phosphorylation of eIF2α, was included as a positive control for phosphorylation of eIF2α. 50B11 cells were either or not pre-treated with IFN for 24h and subsequently infected with PRV-Becker or HSV-1 VR-733 (MOI 1). Lysates were collected at 4hpi and Western blotting was performed for eIF2α and phosphorylated eIF2α (P-eIF2α),

ICP4 (in HSV-1 infected samples) and IE180 (in PRV infected samples). Figure 3.A. shows a substantial phosphorylation of eIF2α in IFN-treated samples infected with HSV-1, but not in PRV-infected cells, corresponding with the clear reduction of ICP4 protein expression and the lack of IE180 protein reduction in IFN-treated samples. To confirm these results, an immunofluorescence staining was performed on mock cells either or not pre-treated with thapsigargin and pre-treated cells infected with PRV or HSV-1 (MOI 1). At 4hpi cells were fixed and stained for phosphorylated eIF2α. Figure 3.B. shows that the thapsigargin treatment effectively stimulates the phosphorylation of eIF2α. In PRV-infected cells a clear reduction was seen compared to both untreated and thapsigargin-treated mock cells. The HSV-1 infected cells however had a reduced level of phosphorylation compared to the thapsigargin treated mock cells, but not to the untreated mock cells. Hence, in 50B11 cells, PRV efficiently counteracts both the IFN-induced and thapsigargin-induced phosphorylation of the translation initiation factor eIF2α.

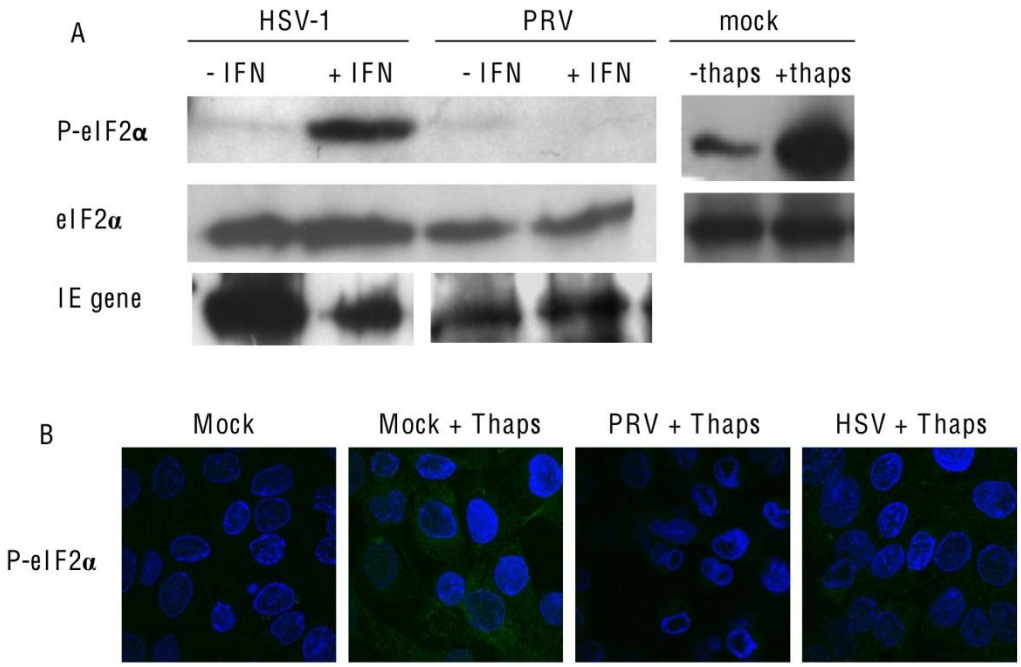


**Figure 3: PRV strongly suppresses IFN-induced phosphorylation of eIF2α.** (A) 50B11 cells were either or not pre-treated with IFN for 24h before virus inoculation. Cell lysates were collected at 4hpi with PRV Becker or HSV-1 VR-733 (MOI 1). Western blotting was performed for total eIF2α, P-eIF2α, IE180 (PRV) and ICP4 (HSV-1). Thapsigargin treatment (1μM for 2h) was included as a positive control to induce phosphorylation of eIF2α. (B) 50B11 cells were either or not pre-treated with thapsigargin (1μM for 1h) before virus inoculation with PRV Becker or HSV-1 VR-733 (MOI 1). Cells were fixed at 4hpi and immunofluorescence staining was performed for P-eIF2α.

## 4. Discussion

The immediate-early (IE) protein ICP4 in HSV-1 and its ortholog IE180 in PRV are the first viral proteins produced during infection of a host cell and are of central importance in the onset of a lytic alphaherpesvirus replication as they act as transactivators of the viral genome (Deluca and Schaffer, 1985). Hence, to enable the establishment of latency in sensory neurons, ICP4/IE180 expression needs to be shut down. Recently, we reported that IFN is able to suppress ICP4 and IE180 protein levels in sensory neurons to undetectable levels, which correlated with the establishment of virus latency in vitro (De Regge et al., 2010). IFN-mediated suppression was less efficient for IE180 compared to ICP4, which correlated with less efficient establishment of in vitro latency for PRV versus HSV-1 (De Regge et al., 2010).

Here, we provide mechanistic insights in the IFN-mediated suppression of ICP4 and IE180 in sensory neuronal cells. We observed that levels of ICP4/IE180 mRNA were mildly but not significantly reduced by IFN at 4hpi, although we did observe a strong ICP4 protein shutdown at that time point. This indicates that IE transcription is not immediately suppressed by IFN in sensory neuronal cells, allowing some ICP4/IE180 mRNA to be produced. At later stages of infection (8hpi and 12hpi), however, we did observe a substantial reduction in ICP4/IE180 mRNA levels in IFN-treated samples, suggesting a transcriptional block at these time points. A transcriptional block of HSV-1 and PRV immediate-early expression has been reported before, although the underlying mechanism remains unknown (Gloger and Panet, 1984; Oberman and Panet, 1988; De Stasio and Taylor, 1990; Nicholl and Preston, 1996; Yao et al., 2007). Several potential explanations could be given to explain such a transcriptional block. On the one hand, IFN may affect expression levels of cellular proteins involved in viral DNA transcription. Initial transcription of alphaherpesviruses is regulated by a transcription complex that comprises the viral VP16 protein and the cellular Oct-1 and HCF1 (Hagmann et al., 1995). It has been reported that IFNα causes a downregulation of Oct-1 and Oct-2 resulting in a repressed transcription (Dent et al., 1991). Other IFN-mediated effects on transcription have mostly focussed on RNA viruses and include IFN-induced proteins like APOBEC3G, TRIM5alpha, and ISG20 (Liu et al., 2010). Whether these or other IFN-induced effectors may also interfere with transcription of DNA viruses will be interesting to further investigate. On the other hand, IFN has been reported to induce activity of RNase L, which may lead to IE mRNA degradation (Liu et al., 2010). Arguing against this possible explanation is our observation that expression of the reference gene GADPH was not reduced in IFN-treated samples (data not shown). This either suggests that the effects of IFN on ICP4/IE180 mRNA cannot be (solely) attributed to IFN-induced RNA degradation or that such degradation is selective towards viral transcripts. For HSV-1, we observed a fast and efficient IFN-induced suppression in ICP4 protein levels

in sensory neuronal cells (4hpi), before substantial shutdown of mRNA levels (from 8hpi onwards). This may indicate that in HSV-1, ICP4 transcripts that are present at 4hpi are not efficiently translated into protein. One of the most potent effects of IFN on translation is phosphorylation and thereby inactivation of the translation initiation factor eIF2α through protein kinase R (PKR) (Liu et al., 2010). Accordingly, in HSV-1-infected sensory neuronal cells, we observed a marked, IFN-induced phosphorylation of eIF2α. For PRV, on the other hand, we did not observe eIF2α phosphorylation. Probably as a result of this, PRV IE180 protein levels were not reduced by IFN at 4hpi. The apparent ability of PRV to counteract phosphorylation of eIF2α and the concomitant inability of IFN to efficiently shut down IE180 protein levels correlate well with our earlier findings that IFN results in less efficient suppression of PRV IE180 compared to HSV-1 ICP4 and concomitant less efficient establishment of PRV versus HSV-1 latency in in vitro cultures of sensory neurons (De Regge et al., 2010). Nevertheless, our finding that HSV-1 is less capable to suppress eIF2α phosphorylation than PRV in sensory neuronal cells was somewhat surprising. Indeed, HSV-1 has been reported to encode a potent factor that interferes with eIF2α phosphorylation, ICP34.5, which functions as an antagonist of the PKR response by redirecting the host protein phosphatase 1α to dephosphorylate eIF2α (He et al., 1997). To exclude the possibility that ICP34.5 was lost by growing virus on cell cultures, ICP34.5 was detected after HSV-1 infection in 50B11 cells (data not shown). It appears that ICP34.5 is less efficient in dephosphorylating eIF2α than PRV (Fig. 3.A. and 3.B.). In cells pre-treated with thapsigargin and subsequently infected with HSV-1 an intermediary level of phosphorylation was observed. The phosphorylation of eIF2α was lower in HSV-1 infected cells pre-treated with thapsigargin than in pre-treated mock cells, indicating that ICP34.5 is able to dephosphorylate eIF2α. The dephosphorylation is PRV infected cells pre-treated with thapsigargin was more efficient (Fig. 3.B.). Despite the fact that, under the current experimental conditions, PRV prevented IFN-induced eIF2α phosphorylation more efficiently than HSV-1, PRV does not encode an ICP34.5 ortholog. Future research will aim at identifying the viral factor(s) involved. An important first clue towards the underlying mechanism of PRV-mediated interference with eIF2α phosphorylation can already be deduced from the current experiments. Both in the absence or presence of IFN, the phosphorylation signal of eIF2α in PRV-infected cells was undetectable and therefore even lower than the basal level observed in mock-infected cells (Fig. 3.A.). This suggests that PRV actively dephosphorylates eIF2α, rather than merely preventing induction of phosphorylation of eIF2α upon IFN treatment. Hence, it is likely that a cellular phosphatase, activated by PRV, is involved.

In conclusion, IFN is able to suppress HSV-1 ICP4 and PRV IE180 levels in sensory neuronal cells by i) reducing the transcription of these viral genes, relatively late in infection and ii) for HSV-1, preventing translation of ICP4 early in infection likely through the phosphorylation of eIF2α. In addition, PRV is able to efficiently inhibit phosphorylation of eIF2α through an unknown mechanism.

## 5. Acknowledgments

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# Chapter 5: Inhibition of histone deacetylases impairs interferon-mediated suppression of HSV-1 ICP4 gene expression

Manuscript in preparation

## Abstract

Interferon (IFN) suppresses mRNA and protein expression levels of immediate-early (IE) genes of alphaherpesviruses like herpes simplex virus (HSV) and may contribute to viral latency in neurons. Although it is unclear how IFN affects viral IE mRNA expression levels, increasing evidence indicates that transcription of alphaherpesviruses is tightly controlled by chromatin modifications. Specifically, the repressive CoREST/REST/HDAC/LSD1 complex controls transcription of the viral genome and may be involved in latency. Here, using different assays (RT-qPCR, Western blot, and immunofluorescence), we show that inhibition of histone deacetylase (HDAC) activity by trichostatin A significantly decreases the ability of IFN to suppress mRNA and protein expression levels of the IE gene ICP4 of HSV in neuronal cells. In addition, IFN substantially upregulated CoREST, a critical component of the CoREST/REST/HDAC/LSD1 transcription repressor complex. Together, these data for the first time indicate that the suppressive effect of IFN on viral replication may involve gene silencing factors, including histone deacetylases.

## 1. Introduction

The human herpes simplex virus type 1 is a common human pathogen that causes disease symptoms that may range from socially discomforting but relatively benign like cold sores and genital lesions, to severe and potentially life-threatening like keratitis, blindness, and encephalitis (Jones, 2003). A critically important characteristic of alphaherpesviruses is their ability to establish lifelong latent infections, generally in sensory neuronal cells. Stress and other triggering factors factors may lead to virus reactivation and recurrent symptoms (Sainz et al., 2001). The transition from a lytic infection to latency is thought to be the result of an interplay between the virus, the neuron and the immune system. Increasing evidence indicates that the switch from lytic to latent and back is regulated by chromatin modifications (Kutluay and Triezenberg, 2009). The CoREST/REST/HDAC/LSD1 repressor complex appears to play a central role in this process (Knipe and Cliffe, 2008; Kutluay and Triezenberg, 2009; Roizman, 2011). This complex may silence the viral genome during some stages of infection, like latency, through the activity of its histone deacetylases (HDAC) that remove the acetylation marks on the genome-associated histones, thereby leading to a transcription suppressing heterochromatin state of the genome (Knipe and Cliffe, 2008; Kutluay and Triezenberg, 2009; Roizman, 2011). On the other hand, this HDAC-dependent silencing complex may be disrupted to allow lytic infection at other stages of infection, like acute infection and reactivation. As an example of the latter, the ICP0 protein of HSV is able to dislodge HDACs from the CoREST/REST/HDAC/LSD1 complex to preserve acetylation and thus open chromatin structure of the viral genome (Hobbs and Deluca, 1999; Poon et al., 2002; Poon et al., 2003).

Over the past years, it has become evident that interferons play a central role in the latency-reactivation cycle of alphaherpesviruses. By suppressing the expression of the earliest viral genes, the immediate-early (IE) genes, interferons contribute to both the establishment and maintenance of alphaherpesvirus latency in neurons (Gloger and Panet, 1984; De Stasio and Taylor, 1990; Nicholl and Preston, 1996; De Regge et al., 2010). Depsite its potential importance for our understanding of alphaherpesvirus latency, the mechanism how IFN suppresses mRNA levels of IE genes like ICP4 of HSV is unclear (De Regge et al., 2010; Van Opdenbosch et al., 2011). Here, we report that the histone deacetylase inhibitor trichostatin A counteracts the IFN-mediated effect on ICP4 gene expression. To our knowledge, this is the first report to suggest IFN may act on the transcription of viral IE genes through histone modifications. In support of such an activity, IFN potently upregulated protein expression of CoREST, an important component of the CoREST/REST/HDAC/LSD1 repressor complex.

## 2. Material and methods

### *2.1. Cells and virus*

Sensory neuronal cells originating from rat dorsal root ganglion neuronal (50B11) cells were a kind gift from Dr. Höke (Department of Neurology, Johns Hopkins University). The cells were grown in neurobasal medium supplemented with 1.1% glucose (20%), 0.27% L-glutamine, 10% fetal calf serum, 2% B-27 and 0.1% blasticidin (Chen et al., 2007). Differentiation of the cells was obtained by treatment with forskolin (50µM) (Sigma) for 24h. HSV-1 strain F (Ejercito et al., 1968) was grown and titrated on Vero cells and stored at -80°C.

### *2.2. Chemical treatments*

Trichostatin (TSA; Sigma) is an HDAC inhibitor. Different concentrations were tested for cytotoxicity and 100nM was found to be the optimal concentration in line with other reports (Pinnoji et al., 2007; Everett et al., 2008). Pre-treatment of 2h prior to infection was performed. Cells were pre-treated with a combination of 1000U/ml universal type I IFN-α (R&D) and 100ng/ml recombinant rat IFN-γ (R&D) for 24h before virus inoculation. IFN remained present during infection.

### *2.3. Infection*

A monolayer of cells was inoculated with HSV-1 strain F at a multiplicity of infection of 1. Cells were fixed or lysed at 8hpi. Before Western blot assays, protein concentrations of all lysates were determined using BCA protein assay reagent according to the manufacturer's instructions (Pierce Biotechnology).

### *2.4. Immunofluorescence staining*

Immunofluorescence staining was performed as described before (Van Opdenbosch et al., 2011). Primary antibody mouse monoclonal anti-ICP4 (Santa Cruz; sc56986), secondary antibody goat-anti-rabbit FITC (Invitrogen) and Hoechst 33342 (Invitrogen) were diluted 1/200 in PBS. Cells were analyzed using an Olympus X81 microscope connected to a Cell*M Imaging Module (Olympus). ICP4 positive cells were counted (5 microscope fields using a 40X magnification lens) and percent reduction in ICP4-positive cells in TSA-treated versus non-treated infected cells upon IFN treatment was calculated. Statistical analysis on four independent replicates was performed using GraphPad Prism software using a t-test ($p < 0.01$).

### *2.5. Western blotting*

SDS/PAGE and Western blotting were performed as described previously (Deruelle et al., 2007). The blots were incubated for 1h or overnight (according to the manufacturer's instructions) with primary antibodies ICP4 (Santa Cruz; sc56986), HDAC1 (Santa Cruz; sc8410), CoREST (Santa Cruz; sc135873), eIF2α (Cell Signaling; L57A5), phospho-eIF2α (Cell Signaling; D9G8), LSD1 (Cell Signaling; C69G12), tubulin (abcam; ab15246) and washed 3 times in 0.1% TBS/Tween-20 (TBS-T). Blots were incubated with HRP-conjugated secondary antibodies (Cell Signaling or Dako Cytomation) for 1h at room temperature and after several washing steps they were developed with enhanced chemiluminescence (ECL Plus; GE Healthcare). Protein band intensities were measured using Image J software. Statistical analysis on four independent replicates was performed using GraphPad Prism software using a t-test ($p < 0.001$).

### *2.6. Quantitative RT-PCR*

RT-qPCR was performed as described before (Van Opdenbosch et al., 2011). RNA samples were prepared as four independent replicates and results of each replicate were normalized to GAPDH. Means and standard deviations were calculated from 4 independent repeats with 2 replicates within each repeat. Subsequently the ratio of IFN-treated versus untreated values was determined. Statistical analysis was performed by using GraphPad Prism software using a t-test ($p < 0.05$).

## 3. Results and discussion

### *3.1. HDAC inhibitor trichostatin results in reduced IFN-mediated suppression of ICP4 mRNA and protein levels*

We previously showed that treatment of 50B11 cells with IFN resulted in suppressed mRNA and protein levels of the immediate-early (IE) gene ICP4 of HSV (Van Opdenbosch et al., 2011). Other studies have also shown a suppressive effect of IFN on IE gene mRNA expression (Gloger and Panet, 1984; De Stasio and Taylor, 1990; Nicholl and Preston, 1996; De Regge et al., 2010), although the underlying mechanism has remained elusive. Alphaherpesvirus transcription is controlled by chromatin modifications, especially histone deacetylases (HDACs) (Kutluay and Triezenberg, 2009). To investigate whether histone deacetylation is involved in the suppressive effect of IFN on ICP4 expression levels, the histone deacetylase inhibitor trichostatin (TSA) was used. Differentiated 50B11 cells were treated or not with IFN for 22h, and subsequently treated or not with 100nM TSA for 2h followed by inoculation with HSV-1. At 8hpi, total RNA was isolated and converted to cDNA

followed by quantitative PCR. The results were normalized to GAPDH and the ratio of IE mRNA levels in IFN-treated versus untreated cell samples was calculated both for cells with or without TSA treatment. Figure 1 shows that mRNA levels of ICP4 were significantly higher in IFN-pretreated cells that were treated with TSA as compared to cells that were mock-treated. In the absence of IFN, TSA had no noticeable effect on ICP4 expression levels (data not shown). These data indicate that HDACs are involved in IFN-mediated reduction of transcription of ICP4.

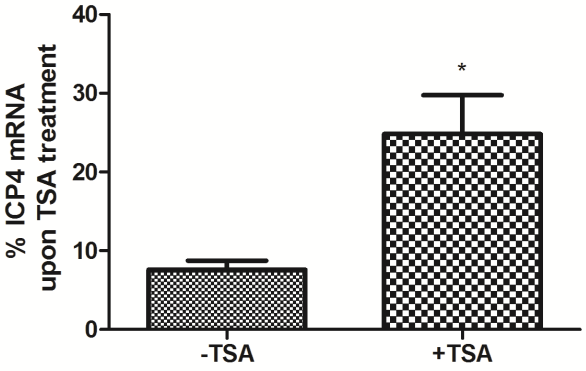


**Figure 1: Trichostatin counteracts IFN-mediated suppression of ICP4 mRNA levels.** Differentiated 50B11 cells were pre-treated with IFN for 22h and subsequently either or not treated with 100nM TSA for 2h prior to inoculation with HSV. At 8hpi, total RNA was isolated and converted into cDNA followed by RT-qPCR for GADPH and ICP4. RNA samples were prepared as four independent replicates and results of each replicate were normalized to GAPDH. Means and standard deviations were calculated and subsequently the ratio of IFN-treated versus untreated values was determined. Asterisk indicates significant difference.

To test whether the effect of TSA on ICP4 mRNA levels resulted in effects on the protein level of ICP4, immunofluorescence and Western blot assays were performed. Earlier reports have shown that simultaneous addition of the HDAC inhibitor TSA and IFN results in suppressed expression of different IFN-inducable genes (Genin et al., 2003; Nusinzon and Horvath, 2003; Chang et al., 2004; Sakamoto et al., 2004; Vlasakova et al., 2007). To avoid any effects of TSA on the induction of IFN-stimulated genes, cells were pre-treated for 22h with IFN before being subjected to a 2h treatment with TSA and virus infection. Differentiated 50B11 cells were treated or not with IFN for 22h, and subsequently treated or not with 100nM TSA for 2h followed by inoculation with HSV-1. At 8hpi, cells were either fixed for immunofluorescence staining or lysed to perform Western blotting for ICP4. Immunofluorescently labelled samples were analysed to determine the percentage of ICP4 positive cells. As shown in figure 2.A., IFN treatment resulted in 31.24% ± 4.5 of ICP4 positive cells that were not treated with TSA, while it resulted in 51.04% ± 1.5 of ICP4 positive cells in cells treated with TSA. Again, in the absence of IFN, TSA had no noticeable effect on the percentage of ICP4-positive cells (data not shown). This indicates that addition of TSA significantly counteracted the suppressive effect of IFN on ICP4 protein expression.

These results were confirmed by Western blotting (Fig.2.B and C). Samples treated with both IFN and TSA showed a significantly higher expression level of ICP4 as compared to IFN alone. Again, as can be seen in the blot, in the absence of IFN, TSA had no noticeable effect on ICP4 protein expression levels. Together, the RT-qPCR data, immunofluorescence and Western blot data show that the IFN-mediated effect on IE transcription involves the activity of histone deacetylases.

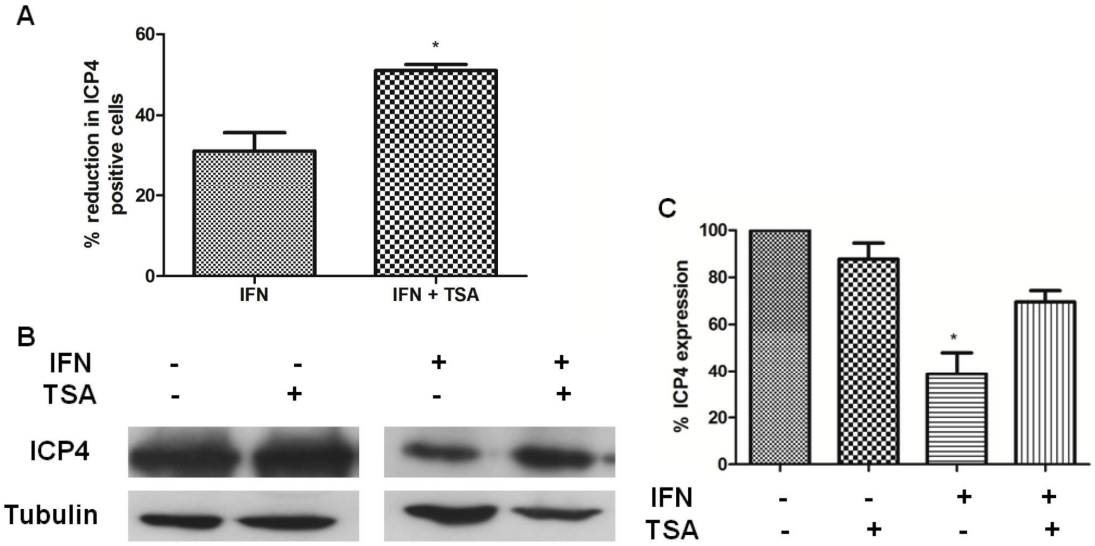


**Figure 2: Trichostatin counteracts IFN-mediated suppression of ICP4 protein levels.** Differentiated 50B11 cells were either or not treated with IFN for 22h. Prior to HSV inoculation, TSA was either or not added to the medium for 2h (100nM). At 8hpi, cells were either fixed and stained or lysed for Western blotting. A) Percentage of ICP4 positive cells after IFN pre-treatment and TSA or mock treatment based on immunofluorescence staining for ICP4. Asterisk indicates significant difference. B) Western blotting for ICP4 with or without IFN pre-treatment and TSA treatment. C) Relative percentage of ICP4 protein, compared to HSV-infected controls (set to 100%). Asterisk indicates significant difference.

Obviously, the fact that TSA treatment does not fully restore ICP4 protein levels to untreated controls (Fig.2.C) is in line with the multiple effects of IFN on viral transcription and translation. For example, IFN-mediated activation of RNaseL, IFN-mediated induction of APOBECs and ADAR1 proteins, and IFN-induced phosphorylation of translation initiation factor eIF2α all have suppressive consequences for protein translation (Bass, 1997; Baumert et al., 2007; Suspene et al., 2011; Van Opdenbosch et al., 2011).

### *3.2. IFN treatment upregulates CoREST*

The alphaherpesvirus genome can be silenced through the CoREST/REST/HDAC/LSD1 repressor complex (Liang et al., 2009; Roizman, 2011). To investigate whether IFN may affect expression levels of one or more components of this repressor complex, which would be in line with the HDAC-mediated effect of IFN on ICP4 expression, protein levels of HDAC1/2, CoREST and LSD1 were determined in differentiated 50B11 cells either mock-treated or treated with IFN for 24h. Figure 3 shows that IFN treatment caused a robust upregulation of CoREST protein levels, without affecting HDAC1/2 and LSD1 protein levels. Although further studies are needed to further explore this, these data suggest that IFN treatment primes the cell to silence the incoming viral genomes by increasing CoREST protein levels.

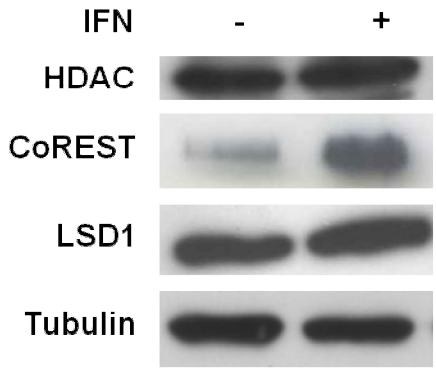


**Figure 3: IFN treatment upregulates CoREST protein levels.** Differentiated 50B11 cells were mock-treated or treated with IFN for 24h. Cells were lysed subsequently and Western blotting was performed for HDAC1/2, CoREST, LSD1 and the loading control tubulin.

The CoREST/REST/HDAC/LSD1 repressor complex is known to play a critical role in silencing of the HSV-1 genome (Gu et al., 2005; Gu and Roizman, 2009b; Gu and Roizman, 2009a; Zhou et al., 2011). REST and CoREST are central players in the transcriptional and epigenetic regulatory circuitry that is responsible for modulating neural genes and they have been implicated in establishing cell identity and function, both within the nervous system and beyond (Qureshi et al., 2010). In a recent study, Du et al. (2010) generated HSV-1 recombinant viruses overexpressing either WT-REST or dnREST to investigate whether HSV-1 hijacks the repressor complex to silence itself. Interestingly, they found that HSV-1 overexpressing WT-REST (but not the strain overexpressing dnREST) was unable to replicate in trigeminal ganglia (TG) of mice, the major site of latency. This indicates that upregulation/overexpression of the REST/CoREST complex may be sufficient to silence HSV-1, a critical step in the establishment of latency (Du et al., 2010). This is entirely in line with our findings. Indeed, we showed before that IFN suppresses ICP4 expression and

drives HSV latency in porcine TG neurons (De Regge et al., 2010). In the current manuscript, we show that IFN upregulates REST's partner CoREST and suppresses HSV ICP4 expression levels through histone modifications, which may interconnect these findings.

Interestingly, we found that IFN upregulates CoREST while protein expression levels of two other central components of the CoREST/REST/HDAC/LSD1 repressor complex, HDAC and LSD1, were not affected. A REST-specific antibody unfortunately did not yield a specific signal. A hypothetical explanation for the difference in modulation of CoREST versus HDAC/LSD1 protein levels in IFN-treated cells perhaps may be that HDAC and LSD1 are constitutively highly expressed in cells, as they are involved in several different cellular complexes, which is the case for CoREST (Bertos et al., 2001; Fischle et al., 2002; Karagianni and Wong, 2007; Mcdonel et al., 2009; Ramirez and Hagman, 2009; Qureshi et al., 2010; Hayakawa and Nakayama, 2011). Further research will clarify this. In line with the findings of Du et al. (2010), we hypothesize that upregulation of CoREST or its partner REST contributes to the silencing of the incoming viral genome.

In conclusion, we report in this study that the IFN-mediated effect on the IE gene transcription is at least partly regulated by HDACs and possibly by the involvement of the CoREST/REST/HDAC/LSD1 repressor complex. This report for the first time suggests that IFN treatment may prime the cell to a viral genome silencing state.

## 4. Acknowledgements

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# Chapter 6: The IE180 protein of pseudorabies virus suppresses phosphorylation of the translation initiation factor eIF2α

Adapted from: N. Van Opdenbosch, N. De Regge, E. Tabarés and H.W. Favoreel. (Submitted)

## Abstract

We have previously shown that the porcine alphaherpesvirus pseudorabies virus (PRV) efficiently interferes with phosphorylation of the eukaryotic translation initiation factor eIF2α. Inhibition of phosphorylation of eIF2α has been reported earlier for the closely related alphaherpesvirus herpes simplex virus type 1 through its ICP34.5 and US11 proteins. PRV however does not encode an ICP34.5 or US11 ortholog. Assays using cycloheximide, UV-inactivated PRV or phosphonoacetic acid (PAA) showed that *de novo* expression of one or more (immediate) early viral protein(s) is required for interference with eIF2α phosphorylation. In line with this, a time-course assay showed that eIF2α phosphorylation was abolished within 2h post PRV inoculation. PRV encodes only one immediate-early protein, IE180, the orthologue of HSV-1 ICP4. A combinational treatment of cells with cycloheximide and actinomycin D, allowing the sole expression of IE180 in PRV-infected cells, led to a substantial reduction in eIF2α phosphorylation levels, indicative for an involvement of IE180. In support of this, transfection of IE180 also potently reduced eIF2α phosphorylation. IE180-mediated interference with eIF2α phosphorylation was not cell type dependent as it occurred both in rat neuronal 50B11 cells and in swine testicle ST cells. Inhibition of the cellular phosphatase PP1 impaired PRV-mediated interference with eIF2α phosphorylation, indicating that PP1 is involved in this process. In conclusion, the immediate-early IE180 protein of PRV has the previously uncharacterized ability to suppress phosphorylation levels of the eukaryotic translation initiation factor eIF2α.

## 1. Introduction

The translation initiation factor eIF2α plays a critical role in the onset of translation of mRNA, including viral mRNA. Phosphorylation of eIF2α prevents recycling of GDP-bound eIF2α into its active GTP-bound form, thereby globally inhibiting protein synthesis (Schneider and Mohr, 2003). Phosphorylation of eIF2α represents one of the potent antiviral consequences of the interferon (IFN)-mediated immune response (Goodbourn et al., 2000). IFN leads to the production of protein kinase PKR, but viral dsRNA is necessary to mediate PKR dimerization and activation. Activated PKR then phosphorylates eIF2α, shutting down translation and viral protein production.

Because of its central importance in ensuring translation of mRNA, different viruses have evolved mechanisms to counteract phosphorylation of eIF2α. We have recently shown that the porcine alphaherpesvirus pseudorabies virus (PRV) very efficiently counteracts phosphorylation of eIF2α (Van Opdenbosch et al., 2011). PRV is closely related to the human herpes simplex virus type 1 (HSV-1). Inhibition of eIF2α phosphorylation has been reported earlier for HSV-1 through its US11 and ICP34.5 proteins (Chou et al., 1995; He et al., 1997; Cassady et al., 1998; Mulvey et al., 1999). US11 binds dsRNA, thereby abrogating its ability to bind PKR, resulting in PKR inactivity and preservation of protein synthesis (Poppers et al., 2000; Cassady and Gross, 2002). The ICP34.5 protein directly causes dephosphorylation of eIF2α through association with the protein phosphatase 1 (PP1) (He et al., 1997). PRV however does not encode an ICP34.5 or US11 ortholog. The aim of the current study was therefore to investigate the mechanism of PRV-mediated dephosphorylation of eIF2α.

In this study, we report that both in rat 50B11 neuronal cells and swine ST cells, *de novo* synthesis of the immediate-early protein IE180 of PRV is able to interfere with eIF2α phosphorylation in 50B11 rat neuronal cells and ST swine testicle cells. We also show that the cellular protein phosphatase 1 is involved in this process although IE180 does not upregulate protein expression levels of the PP1/GADD34 complex.

## 2. Materials and Methods

### *2.1. Cells and virus*

Sensory neuronal cells originating from rat dorsal root ganglion neurons (50B11) cells were a kind gift from Dr. Höke (Department of Neurology, Johns Hopkins University). The cells were grown in neurobasal medium supplemented with 1.1% glucose (20%), 0.27% L-glutamine, 10% fetal calf serum (FCS), 2% B-27 and 0.1% blasticidin (Chen et al., 2007). Before use in experiments, cells were differentiated as described before (Chen et al., 2007; Van Opdenbosch et al., 2011), by treatment with forskolin (50μM) (Sigma) for 24h. Swine Testicle (ST) cells were cultivated in Eagle's minimal essential medium (MEM) supplemented with 10% FCS, glutamine (0.3 mg/ml) and antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin). The PRV strain Becker (Card et al., 1990) was grown and titrated on ST cells and stored at -80°C.

### *2.2. Antibodies and chemicals*

The rabbit polyclonal anti-IE180 antibody has been described before (Gomez-Sebastian and Tabares, 2004). Mouse monoclonal anti-US3 antibody was kindly provided by L.A. Olsen and L. Enquist (Princeton University, USA). Mouse anti-eIF2α (L57A5), rabbit anti-phospho-eIF2α (D9G8), rabbit anti-phospho-PERK (16F8) and rabbit anti-PP2A (52F8) were purchased from Cell Signaling. Mouse anti-PP1 (sc-7482) and rabbit anti-GADD34 (sc-8327) were purchased from Santa Cruz. Rabbit anti-GFP (G10362) was purchased from Invitrogen and rabbit anti-alpha tubulin (ab15246) was purchased from Abcam. HRP-conjugated secondary goat anti-rabbit was purchased from Cell Signaling and goat anti-mouse antibodies were purchased from Dako Cytomation.

To induce phosphorylation of eIF2α, cells were treated with 1μM thapsigargin (Invitrogen) for 1h. Thapsigargin induces eIF2α phosphorylation by activating the pancreatic endoplasmic reticulum PERK protein kinase (Wong et al., 1993). Cycloheximide (CHX, Sigma-Aldrich) treatment (10μg/μl) for 30min prior to inoculation was used to inhibit protein translation and phosphonoacetic acid (PAA, Sigma-Aldrich) treatment (250μg/ml) was used to inhibit viral replication. When ultraviolet (UV) inactivated PRV was used, inactivation was performed by UV irradiating the inoculum in a petri dish on ice with 1000mJ/cm$^2$ using the CL-1000 UV Crosslinker (UVP, Inc.), all as described before (Deruelle et al., 2009). Sequential treatment with cycloheximide and actinomycin D (ActD, Invitrogen) was used to allow expression of the immediate-early protein IE180, as described before (Ambagala et al., 2000). Briefly, cells were treated with CHX (10μg/μl) for 30min prior to inoculation. At 2hpi (hours post inoculation), CHX was washed away and medium with ActD (5μg/ml) was added.

PP1- and PP2 inhibitors inhibitor 2 (I2) and okadaic acid (OA) were obtained from Millipore and Sigma-Aldrich respectively, and used at concentrations recommended by the manufacturer (I2) or found in literature (Wang et al., 2009). Cells were treated for 1h prior to inoculation with 0.2µM I2 or 20nM OA to inhibit cellular phosphatases PP1 or PP2 respectively.

### *2.3. Infection and transfection*

For infection assays, monolayers of cells, pre-treated with thapsigargin (1µM for 1h) were inoculated with the PRV strain Becker at a multiplicity of infection (MOI) of 1. Cells were lysed for immunoblotting at 4hpi. For transfection assays, subconfluent 50B11 cells were transfected with an IE180 expressing plasmid (Munoz et al., 2010) or an eGFP expressing plasmid (pTrip, obtained from B. Verhasselt, Ghent University) using the jetPRIME$^{TM}$ reagent (Polyplus) according to the manufacturer's protocol. Medium was renewed 4h post transfection. Cells were treated with thapsigargin (1µM for 1h) prior to lysis 24h post transfection.

### *2.4. Western blotting*

SDS/PAGE and Western blotting were performed as described previously (Deruelle et al., 2007). Protein concentrations of 30-40µg were used for all experiments. Protein concentration was determined using the BCA protein assay reagent and spectrophotometry according to the manufacturer's instructions (Pierce Biotechnology). Blots were blocked in 5% non-fat dry milk in PBS/Tween-20 for 1h at room temperature. The blots were incubated for 1h or overnight (according to the manufacturer's instructions) with primary antibodies and washed 3 times in 0.1% TBS/Tween-20 (TBS-T). Blots were incubated with HRP-conjugated secondary antibodies for 1h at room temperature and, after several washing steps, developed using enhanced chemiluminescence (ECL Plus; GE Healthcare). Protein band intensities were measured using Image J software. Statistical analysis on three independent replicates was performed using GraphPad Prism software using a t-test.

# 3. Results

## *3.1. PRV interferes with eIF2α phosphorylation*

We previously described that PRV is able to counteract IFN-mediated phosphorylation of eIF2α in 50B11 neuronal cells (Van Opdenbosch et al., 2011). Here, we used thapsigargin to induce phosphorylation of eIF2α. Thapsigargin induces eIF2α phosphorylation by activating the pancreatic endoplasmic reticulum PERK protein kinase (Wong et al., 1993). Differentiated 50B11 cells were pre-treated or not with thapsigargin (1µM) for 1h prior to inoculation with PRV. Lysates were collected at 4hpi and Western blotting was performed for total and phosphorylated eIF2α. Thapsigargin treatment effectively induced phosphorylation of eIF2α (Fig. 1A, lanes 1-2, Fig. 1B). PRV efficiently counteracted phosphorylation of eIF2α in thapsigargin-stimulated cells and also reduced basal levels of eIF2α phosphorylation in non-stimulated cells (Fig. 1A, lanes 3-4, Fig. 1B). This indicates that PRV is able to interfere with phosphorylation of eIF2α induced by different stimuli. Since PRV is able to counteract both pre-induced (by thapsigargin) and basal levels of phosphorylation of eIF2α, this suggests that PRV leads to dephosphorylation of eIF2α rather than preventing phosphorylation of eIF2α. In support of the latter, PRV did not influence thapsigargin-induced phosphorylation of PERK, the kinase that phosphorylates eIF2α upon thapsigargin treatment (Fig. 1A).

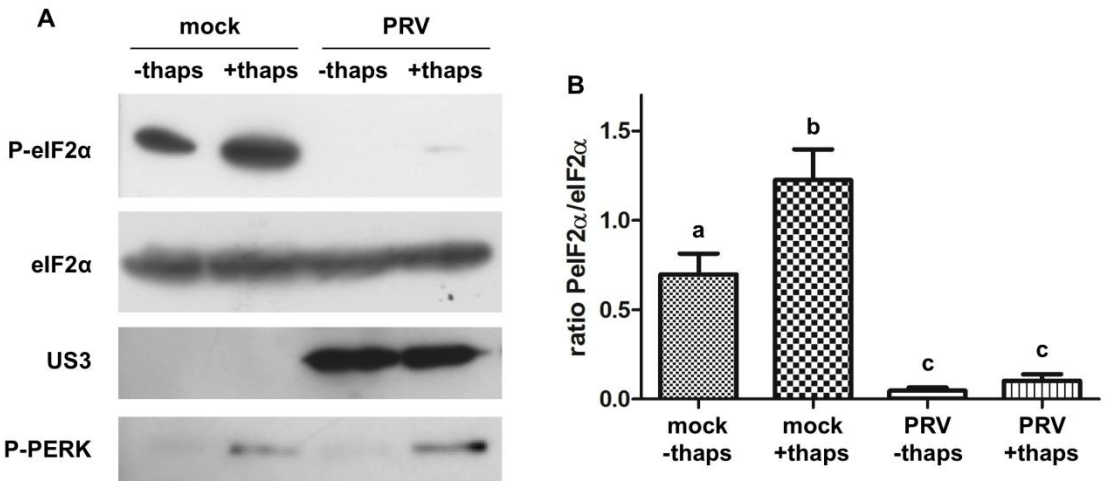


**Figure 1. PRV suppresses phosphorylation of eIF2α.** (A) Differentiated 50B11 cells were pre-treated or not with thapsigargin (1µM) for 1h prior to mock inoculation or inoculation with PRV (MOI 1). Lysates were collected at 4hpi and Western blot detection was performed for total and phosphorylated eIF2α and phosphorylated PERK. US3 detection was included as a control for infection. (B) shows ratios of PeIF2α/total eIF2α of three independent replicates. Different letters indicate statistically significant differences ($p < 0.05$).

### *3.2. An (immediate) early protein is involved in PRV-mediated interference with eIF2α phosphorylation*

For HSV-1, it has been described that the infected-cell protein 34.5 (ICP34.5) triggers dephosphorylation of eIF2α by recruiting the cellular protein phosphatase 1 (PP1) to a multiprotein complex (He et al., 1997). PRV, however, does not encode an ortholog of ICP34.5. To elucidate which viral protein(s) is responsible for interference with eIF2α phosphorylation in PRV-infected cells, we first determined at which stage of infection interference with eIF2α phosphorylation occurs. To investigate whether *de novo* viral protein synthesis is required, thapsigargin-stimulated cells were either treated with cycloheximide (CHX), a translation inhibitor, before and during virus inoculation or were inoculated with UV-inactivated PRV. Cell lysates were collected at 4hpi and Western blot detection was performed for total and phosphorylated eIF2α. Both in PRV-infected cells treated with CHX or cells inoculated with UV-inactivated PRV, the level of phosphorylation of eIF2α was comparable to mock-infected cells, indicating that viral protein synthesis is required for interference with eIF2α phosphorylation (Fig. 2A&B). Phosphonoacetic acid (PAA) inhibits viral replication and thus late viral protein expression. PAA did not affect PRV-mediated interference with eIF2α phosphorylation (Fig. 2C), implicating that one or more (immediate) early viral protein(s) are involved.

In order to confirm these findings, a time-course experiment of PRV infection was performed to determine the time-point of suppression of eIF2α phosphorylation. Levels of phosphorylated eIF2α were determined at 30min, 1h, 2h, 3h and 4hpi. Figure 2D shows that a reduction in phosphorylation of eIF2α is already evident at 1hpi. From 2hpi onwards, phosphorylation levels were very strongly reduced. Expression of the PRV immediate-early protein IE180 was first detected at 1hpi, while substantial expression of the early viral protein US3 was detected from 2hpi onwards. These data indicate that PRV interference with eIF2α phosphorylation occurs very early in infection and involves the expression of one or more immediate-early or early viral proteins.

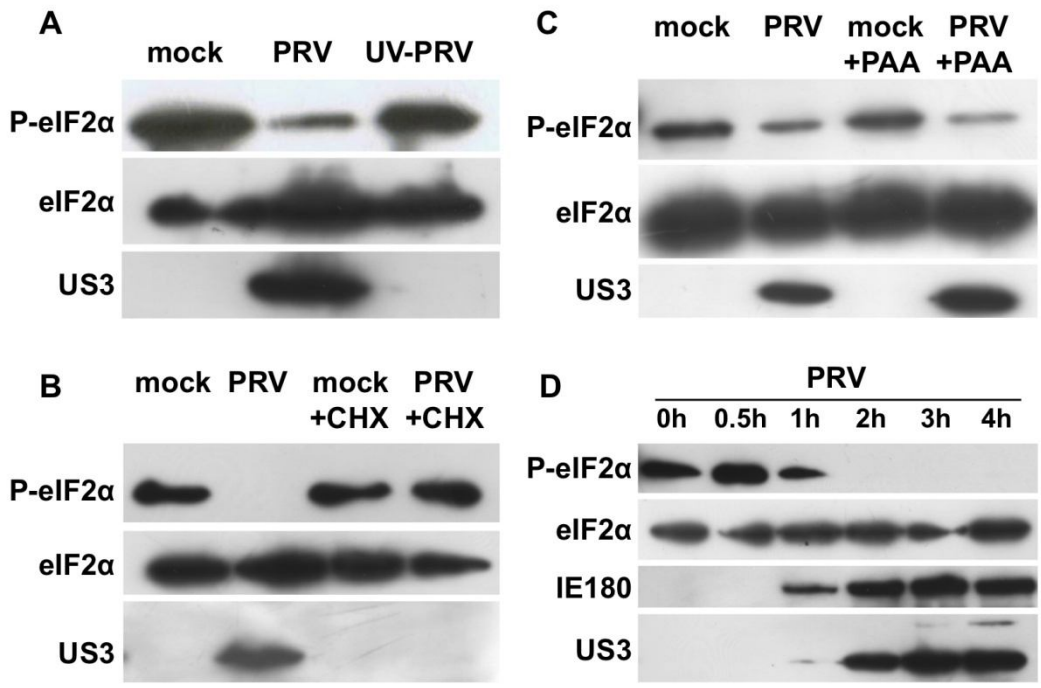


**Figure 2. An (immediate) early protein is involved in PRV-mediated suppression of eIF2α phosphorylation.** (A, B, C) Differentiated 50B11 cells were pre-treated with thapsigargin (1μM for 1h). Subsequently, cells were mock-inoculated or inoculated with UV-inactivated PRV (theoretical MOI 1)(A) or treated with cycloheximide (10μg/μl)(B) or PAA (250μg/ml) (C) prior to inoculation with PRV (MOI 1). Lysates were collected at 4hpi and Western blot detection was performed for total and phosphorylated eIF2α. US3 detection was included as a control for treatment and infection. (D) Differentiated 50B11 cells were pre-treated with thapsigargin (1μM for 1h). Subsequently, cells were inoculated with PRV (MOI 1). Lysates were collected at different time points post inoculation and Western blot detection was performed for total and phosphorylated eIF2α, the immediate early protein IE180, and the early protein US3.

### *3.3. Expression of IE180 interferes with eIF2α phosphorylation*

PRV encodes only one protein with immediate-early kinetics, immediate-early protein 180 (IE180), an ortholog of HSV-1 ICP4. To investigate the possible involvement of IE180, first, a sequential treatment of CHX and actinomycin D (ActD) was used. This treatment results in protein expression of viral genes with immediate-early characteristics (transcribed without *de novo* viral protein synthesis) (Hayes and Rock, 1990; Debrus et al., 1995; Ambagala et al., 2000). In the case of PRV, this results in expression of IE180, the only viral gene product with immediate-early characteristics (Ambagala et al., 2000). At 4hpi, lysates were collected and Western blot detection was performed for total and phosphorylated eIF2α. Detection of IE180 and the absence of detectable early protein US3 were used as controls for the treatment. As shown in Figure 3A&B, phosphorylation of eIF2α is significantly reduced under these circumstances, indicating that the expression of IE180 interferes with eIF2α phosphorylation.

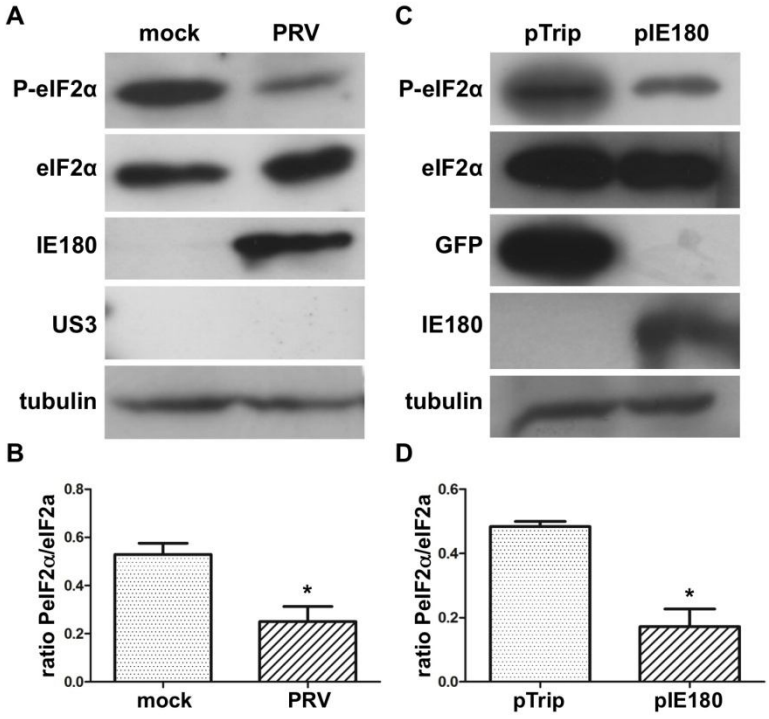


**Figure 3. Expression of IE180 causes suppression of eIF2α phosphorylation.** (A, B) Differentiated 50B11 cells were pre-treated with thapsigargin (1µM for 1h). Subsequently, cells were treated with cycloheximide (10µg/µl) prior to mock inoculation or inoculation with PRV (MOI 1). At 2hpi, CHX was washed away and actinomycin D (5µg/ml) was added to the cells for the remainder of the experiment. At 4hpi, lysates were collected and Western blot detection was performed for total and phosphorylated eIF2α. IE180 and US3 detection were included as control for treatment and tubulin detection was included as a loading control. (B) shows ratios of P-eIF2α/total eIF2α of three independent replicates. (C, D) Differentiated 50B11 cells were transfected with a eukaryotic expression vector encoding IE180 (pIE180) or eGFP (pTrip) for 24h. Upon stimulation with thapsigargin (1µM for 1h), lysates were collected and Western blot detection was performed for total and phosphorylated eIF2α. GFP and IE180 detection were included as transfection control and tubulin detection as loading control. (D) shows ratios of P-eIF2α/total eIF2α of three independent replicates. Asterisks indicate statistically significant differences ($p < 0.05$).

To confirm a potential involvement of IE180, phosphorylation of eIF2α was assessed in 50B11 cells transfected with a eukaryotic expression vector encoding either IE180 or (as a control) eGFP. At 24h post transfection, lysates were collected and Western blot detection was performed for total and phosphorylated eIF2α. Figure 3C&D show that, in samples containing IE180-transfected cells, phosphorylation of eIF2α was significantly diminished. The degree of reduction corresponded well with the transfection efficiency of ± 40%. In conclusion, expression of IE180 protein is sufficient to interfere with phosphorylation of eIF2α.

### *3.4. PRV IE180-mediated suppression of eIF2α phosphorylation also occurs in swine ST cells*

To determine whether our observations hold true in other cells, particularly swine cells, key experiments were repeated in ST swine testicle cells. A time course infection experiment showed rapid suppression of eIF2α phosphorylation in ST cells, within 2hpi, similar compared to 50B11 cells (data not shown). Also, like in 50B11 cells (Fig. 3A), subjection of ST cells to a sequential treatment with CHX and ActD that leads to IE180 protein expression during PRV infection again resulted in a substantial reduction in eIF2α phosphorylation (Fig. 4A). In addition, like in 50B11 cells (Fig. 3B), transfection of IE180 in ST cells resulted in substantial interference with eIF2α phosphorylation that corresponded well with the ± 30% transfection efficiency (Fig. 4B).

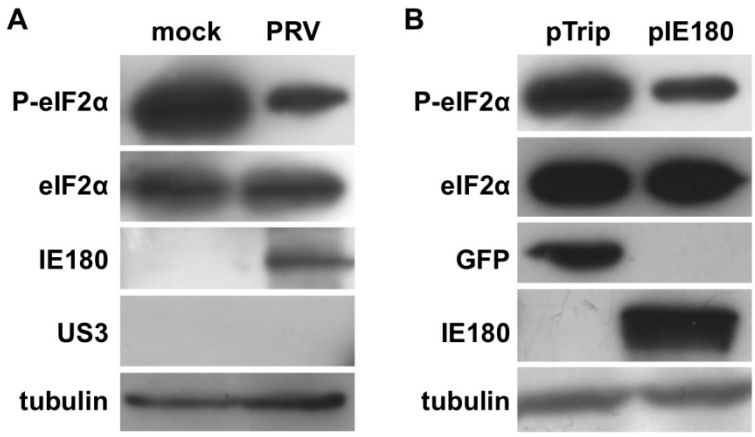


**Figure 4. PRV IE180-mediated suppression of eIF2α phosphorylation in ST cells.** (A) ST cells were pre-treated with thapsigargin (1μM for 1h). Subsequently, cells were treated with cycloheximide (10μg/μl) prior to mock inoculation or inoculation with PRV (MOI 1). At 2hpi, CHX was washed away and actinomycin D (5μg/ml) was added to the cells for the remainder of the experiment. At 4hpi, lysates were collected and Western blot detection was performed for total and phosphorylated eIF2α. IE180 and US3 detection were included as control for treatment and tubulin detection was included as a loading control. (B) ST cells were transfected with a eukaryotic expression vector encoding IE180 (pIE180) or eGFP (pTrip) for 24h. Upon stimulation with thapsigargin (1μM for 1h), lysates were collected and Western blot detection was performed for total and phosphorylated eIF2α. GFP and IE180 detection were included as transfection control and tubulin detection as loading control.

### *3.5. Protein phosphatase 1 but not protein phosphatase 2 is involved in PRV-mediated interference with eIF2α phosphorylation*

As described higher, the observations that PRV suppresses both basal and thapsigargin pre-induced levels of eIF2α phosphorylation and does not affect thapsigargin-induced phosphorylation of PERK (Fig. 1) suggest that PRV leads to dephosphorylation of

eIF2α rather than preventing phosphorylation of eIF2α. This would imply the involvement of a phosphatase. PRV does not encode a viral phosphatase, suggesting that a cellular phosphatase may be recruited or induced by PRV to induce dephosphorylation of eIF2α. Both protein phosphatase 1 (PP1) and protein phosphatase 2 (PP2A) have been reported to dephosphorylate eIF2α (Ernst et al., 1982). To investigate whether PP1 and/or PP2A are involved in PRV-mediated interference with eIF2α phosphorylation, assays were done in the presence of 0.2μM I2 to inhibit PP1 activity or 20nM OA to inhibit PP2 activity. At 4hpi, lysates were collected and immunoblotting was performed for total and phosphorylated eIF2α. Treatment with I2 significantly counteracted PRV-mediated interference with eIF2α phosphorylation while treatment with OA had no effect (Fig. 5A&B). These results indicate that PP1 is involved in PRV-mediated dephosphorylation of eIF2α.

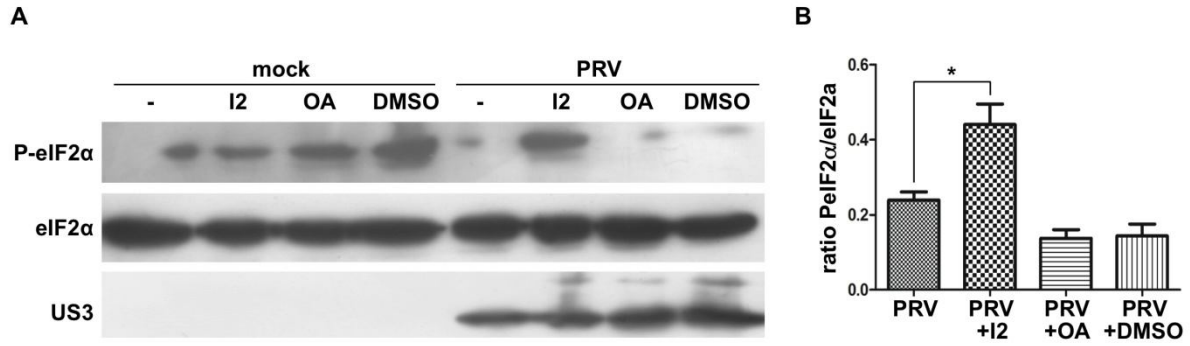


**Figure 5. Protein phosphatase 1 but not 2 is involved in PRV-mediated suppression of eIF2α phosphorylation.** (A) 50B11 cells were pre-treated with thapsigargin (1μM for 1h). Prior to and during inoculation with PRV (MOI 1), cells were treated with PP1 specific inihibitor I2 (200nM), PP2-inhibiting okadaic acid (OA 20nM) or a DMSO control. At 4hpi, lysates were collected and Western blotting was performed for total and phosphorylated eIF2α. US3 detection was included as control for infection. (B) shows ratios of P-eIF2α/total eIF2α of three independent replicates. Asterisk indicates statistically significant difference ($p < 0.05$).

### *3.6. PRV infection or IE180 transfection does not affect PP1 or GADD34 protein expression levels*

Since IE180 is a transactivator, one possibility could be that IE180 induces protein expression of PP1 or its regulatory complex partner GADD34, thereby promoting dephosphorylation of eIF2α. To investigate this, thapsigargin-stimulated 50B11 cells were either mock-infected or infected with PRV or transfected with a eukaryotic expression vector either encoding IE180 or, as a control, eGFP. Western blot detection at 4hpi or 24h post transfection was performed for PP1, PP2A and GADD34 and IE180/US3/tubulin detection was included as control. As shown in Figure 6, neither PRV infection nor IE180 transfection affected PP1, PP2A or GADD34 protein levels. These findings indicate that IE180 does not alter protein expression levels of PP1 or GADD34, to initiate dephosphorylation of eIF2α.

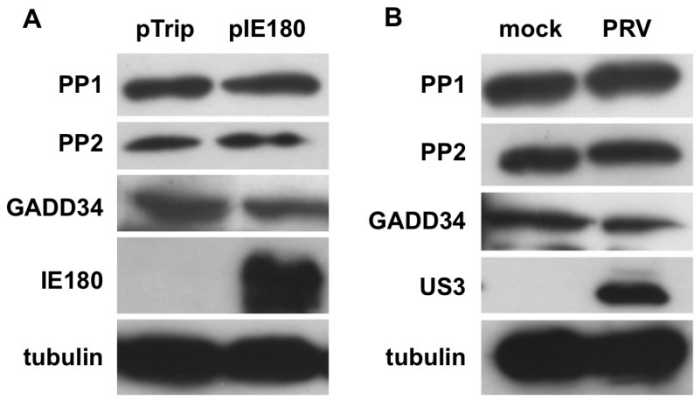


**Figure 6. PRV infection or IE180 transfection does not affect protein expression levels of PP1, PP2, or GADD34.** (A) Differentiated 50B11 cells were transfected with pIE180 or the control plasmid pTrip for 24h and subsequently pre-treated with thapsigargin (1μM for 1h). Lysates were collected and subjected to Western blot detection of PP1, PP2 and GADD34. IE180 and tubulin detection were included as controls. (B) Differentiated 50B11 cells were pre-treated with thapsigargin (1μM for 1h) and subsequently mock inoculated or inoculation with PRV (MOI 1). Lysates were collected at 4hpi and Western blot detection was performed for PP1, PP2 and GADD34. US3 and tubulin detection were included as controls.

## 4. Discussion

Phosphorylation of the translation initiation factor eIF2α results in shutdown of translation, and represents one of the key antiviral effector mechanisms of interferon (Randall and Goodbourn, 2008). Here, we show that the PRV immediate-early protein IE180 interferes with phosphorylation of eIF2α. We also show that the cellular phosphatase PP1 is involved in PRV-mediated interference with eIF2α phosphorylation.

PRV IE180 is the ortholog of HSV-1 ICP4 and VZV IE62. Before the current report, three alphaherpesvirus proteins had been reported to interfere with phosphorylation of eIF2α: ICP34.5 and US11 of HSV-1 (neither of which have orthologs in PRV) and IE63 of VZV (the putative homolog/ortholog of HSV-1 ICP22 and PRV RSp40/ICP22). ICP34.5 and US11 of HSV-1 counteract phosphorylation of eIF2α via different mechanisms. US11 binds to PKR, preventing its activation and thus phosphorylation, while ICP34.5 recruits PP1 to eIF2α to mediate its dephosphorylation (He et al., 1997; Cassady and Gross, 2002; Li et al., 2011). The mechanism underlying VZV immediate-early protein IE63-mediated dephosphorylation of eIF2α is unknown (Ambagala and Cohen, 2007). However, since activation of PKR does not appear to be significantly affected in VZV infected cells (Desloges et al., 2005), PKR probably is not involved in IE63-mediated dephosphorylation of eIF2α. Since we found that PRV suppresses basal and thapsigargin pre-induced phosphorylation of eIF2α, this suggests that IE180 dephosphorylates eIF2α, rather than preventing its phosphorylation, suggestive for the involvement of a phosphatase. In this way, the mode of action of IE180 appears to

resemble ICP34.5 more than US11. In support of this, like for ICP34.5, we showed an involvement of PP1.

Since phosphorylation of eIF2α and subsequent shutdown of protein synthesis brings the entire viral replication in jeopardy, it would appear to make sense if a virus tries to interfere with this system soon upon infection of a host cell. Our finding that the only immediate-early protein encoded by PRV, IE180, interferes with phosphorylation of eIF2α fits with this assumption. VZV also interferes with phosphorylation through a protein with immediate-early kinetics, IE63 (the putative homolog/ortholog of HSV-1 ICP22 and PRV RSp40/ICP22)(Ambagala and Cohen, 2007). Perhaps surprisingly, both HSV-1 proteins that interfere with eIF2α phosphorylation, ICP34.5 and US11, are reported to be late proteins (He et al., 1997; Cassady and Gross, 2002; Bryant et al., 2008). However, at least for ICP34.5, this late expression appears to be leaky, and low amounts of ICP34.5 protein can already be detected 3h post inoculation (Pasieka et al., 2006). These low amounts of ICP34.5 early in infection are sufficient to generate an effect on eIF2α phosphorylation (Pasieka et al., 2006).

It will be interesting to determine in future research whether the ability of PRV IE180 to interfere with phosphorylation of eIF2α is conserved in other alphaherpesviruses. The overall amino acid homology between IE180 orthologs is relatively low, ranging from 32% to 40% in HSV ICP4, VZV IE62, bovine and equine ICP4. However, ClustalW analysis showed that amino acid homology is condensed in 4 highly conserved regions over the different alphaherpesviruses. Hence, it will be interesting to investigate whether other IE180 orthologs affect eIF2α phosphorylation and, if so, to analyze the involvement of these four conserved domains.

How does IE180 affect phosphorylation levels of eIF2α? IE180 is predominantly located in the nucleus, acting as a transactivator to control expression of different genes. From this viewpoint, one potential explanation for the ability of IE180 to interfere with the phosphorylation status of eIF2α could be that IE180 affects gene expression of cellular factors that influence the phosphorylation status of eIF2α. Although we provide evidence that the cellular phosphatase PP1 is involved in IE180-mediated dephosphorylation of eIF2α, IE180 did not affect protein expression levels of either PP1 (or PP2) or its regulatory complex partner GADD34. Future research will show whether IE180 may affect expression of other components that direct eIF2α dephosphorylation. For example, overexpression of the cellular protein CReP has been reported to promote dephosphorylation of eIF2α through recruitment of PP1 (Jousse et al., 2003). Alternatively, it is possible that IE180, upon its production in the cytoplasm, physically interacts with one or more cellular components affecting phosphorylation of eIF2α, thereby modulating their function. Cytoplasmic IE180 has been

detected in PRV-infected cells (Huang et al., 1997). Such putative interaction of IE180 with cellular components affecting eIF2α phosphorylation would be in line with mechanisms employed by different other viruses. For example, ICP34.5 of HSV-1 displays sequence homology to both PP1 and eIF2α. This allows ICP34.5 to serve as a molecular bridge, thereby mediating eIF2α dephosphorylation (He et al., 1997; He et al., 1998; Li et al., 2011). Although we did not find any obvious sequence homology between IE180, eIF2α or PP1, we cannot exclude a similar function for IE180 at this stage and will further address this in future experiments.

In conclusion, we report that the PRV immediate-early protein IE180 has the previously uncharacterized ability to suppress phosphorylation of eIF2α, which may have important consequences for the virus during early stages of virus replication, in the face of an active innate immune response.

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# Chapter 7: General discussion

Herpesviruses are among the most successful pathogens as they are able to establish lifelong latent infections from which they may reactivate to spread to new hosts. Their delicate balance with the host immune system is key to their success. Interferon (IFN) is one of the major components of the host defense against viruses in general and herpesviruses in particular. Indeed, defects in the IFN system may lead to aggravated and sometimes fatal herpesvirus disease (Al-Khatib et al., 2002; Al-Khatib et al., 2003; Vollstedt et al., 2004; Casrouge et al., 2006; Zhang et al., 2007; Zhang et al., 2008; Perez De Diego et al., 2010).

The closely interacting dance, or battle, between IFN and alphaherpesviruses has already been extensively studied, identifying several IFN-mediated effects that control viral replication and viral escape mechanisms. Type I IFNs are among the first immune effectors produced upon alphaherpesvirus infection, playing a key role in limiting replication at the initial stage of infection (Hendricks et al., 1991; Mikloska et al., 1998; Mikloska and Cunningham, 2001; Sainz and Halford, 2002; Jones, 2003). These innate interferons are present both at the periphery and in the trigeminal ganglion (TG) at the time when latency is established (Hendricks et al., 1991; Carr et al., 1998). However, the role of type I IFNs in the switch between lytic and latent infection had not yet been described. To investigate this, we made use of a two-chamber *in vitro* model. This *in vitro* model allows infection of trigeminal ganglion (TG) neurons via the nerve endings in the outer chamber, whereafter the virion is transported retrograde to the nucleus in the inner chamber where replication can occur, much like the infection route *in vivo* (De Regge et al., 2006). Our data indicate that addition of IFNα is sufficient to instigate the switch from lytic to latent infection at least in primary TG neurons in an *in vitro* system.

In the absence of exogenous added IFN, the vast majority of the accessible neurons with axons growing in the outer chamber proceeded to productive infection upon PRV inoculation, correlating with previous results that were obtained without the two-chamber system (Geenen et al., 2005). However, this seems at odds with earlier reports, which suggested that latency establishment may be a default pathway of alphaherpesvirus infection in neurons. An important reason for this belief was the assumption that tegument protein VP16, which is a transactivator required for the onset of lytic replication, might be lost during virion transport along the axon (Roizman and Sears, 1987; Kristie and Roizman, 1988). However, using an inducible VP16-mutant HSV-1, this hypothesis was contradicted, showing that latency was established even in the presence of a functional VP16 (Sears et al., 1991). Another theory posed that sensory neurons lack Oct1 transcription factors, leading to default establishment of latency in neurons (He et al., 1989). However, Hagmann et al. (1995) showed that all components necessary for onset of transcription are present in sensory

neurons, although at lower levels (Hagmann et al., 1995). Later on, it was shown that in neurons, HCF1 is sequestered to the cytoplasm as opposed to the general nuclear distribution in other cell types (Kristie et al., 1995; Kristie et al., 1999; La Boissiere et al., 1999). As HCF-1 is required to recruit VP16 to the nucleus, it might be that this does not occur (efficiently) in sensory neurons (La Boissiere et al., 1999). Although our data show that, at least for PRV, latency does not appear to be the default pathway of infection in TG neurons, some side-remarks have to be made. A potentially important difference between the *in vivo* TG neurons and our *in vitro* system is the length of axons and thus transport distance for the virion. In the *in vitro* model, the virus only has to overcome 1 - 2cm while *in vivo* the virus has to travel at least 10cm, which may for example affect the VP16 protein amounts reaching the nucleus. Also, *in vitro* culture of TG neurons may affect expression levels and/or subcellular localization of transcription factors such as HCF1 and Oct-1. It might be interesting to look at the presence and localization of HCF1, Oct-1, and VP16 in the *in vitro* model to further explore this. Another big difference between the *in vivo* situation and the *in vitro* model is the presence of cytokines and other immune factors. To give just one example, dendritic cells are not present in the *in vitro* cultures whereas these cells have been shown to produce IFNα in response to HSV-1 (Ghanekar et al., 1996) and IFNα has been implicated in restricting viral HSV-1 replication in the cornea (Hendricks et al., 1991). We have shown that addition of IFNα is sufficient to switch alphaherpesvirus infection from lytic to latent in the *in vitro* model, but different other cytokines have been detected in mouse TG carrying latent HSV-1, including IL2, IL6, IL10 and IFNγ (Halford et al., 1996). A very recent study strongly suggests that TGFβ signaling, which generally functions to dampen immune responses, results in increased HSV-1 latency (Allen et al., 2011). Thus it is possible, and even likely, that some of these cytokines affect the process of latency establishment and/or maintenance. Hence, the loss of immune factors in the *in vitro* model has to be taken into account when interpreting results.

While we found 98% of accessible neurons positive for productive PRV infection in the absence of IFN, this percentage was much lower for HSV-1 (12%). Possible explanations are that HSV-1 has difficulties in infecting porcine cells or specifically porcine TG neurons. Previous studies showed that HSV-1 is able to infect porcine cells although the infection efficiency was 1000fold lower in porcine kidney PK15 cells than in monkey Vero cells (Kim et al., 2010). We found that in ST cells (swine testicle cells), only 5-10% of cells were positive for gD at 15hpi with HSV-1 compared to 100% in PRV infected cells, using equivalent infectious doses (data not shown), suggesting a general lower ability of HSV-1 to productively infect porcine cells. To investigate whether the roadblock towards productive HSV-1 infection is situated at the entry level or later in infection, a mutant HSV-1 carrying the

LacZ gene under control of the human CMV MIEP promoter was used in the *in vitro* model. This virus generated detectable β-galactosidase activity in 90% of accessible neurons, indicating that HSV-1 efficiently enters and delivers its DNA in the nucleus of porcine TG neurons. Another possibility is that HSV-1 productive infection is hampered early in infection. In support of this, we found the percentage of neurons positive for the immediate-early protein ICP4 to be similar to the percentage of neurons positive for the late protein gD later in infection, indicating that HSV-1 is able to trigger immediate early viral protein expression and subsequent productive infection in only a small part of the TG neurons whereas the others are halted very early in infection. A recent report from Bertke et al. (2011) showed that in mice, a subpopulation of primary TG neurons, A5-positive neurons, appear to be non-permissive for HSV-1 productive infection. In these $A5^+$ cells, latency was induced immediately upon entry of the cell. Although $A5^+$ neurons make up only 11% of an adult TG, it limits HSV-1 replication as their results showed a maximum of 22% neurons positive for HSV-1 infection at a high MOI (Bertke et al., 2011). The same study showed that the block occurred at or before the IE level as only 2.1% of $A5^+$ neurons were positive for ICP0 and also early and late proteins were present in very low amounts. However there is currently no clear explanation on why this seems to happen (Bertke et al., 2011). Hence, it may be that HSV-1, but not PRV, is only able to initiate productive infection in a subpopulation of TG neurons. Numerous alternative hypothetical explanations may be put forward to explain the substantially lower ability of HSV-1 to initiate productive infection in porcine TG neurons compared to PRV. For example, it might be that, because of the species difference, the VP16/HCF1 interaction may be less efficient or strong in HSV-1 infected porcine TG neurons compared to PRV, resulting in a lower transcription efficiency. It might also be that the CoREST/REST/HDAC/LSD1 repressor complex may be recruited faster and/or more efficient to HSV-1 genomes than to PRV genomes, thereby silencing the genome in a more efficient manner. Yet another possibility may be that VP16 is more tightly associated with the capsid in PRV versus HSV-1, which may lead to higher levels of VP16 reaching the nucleus, allowing more efficient onset of productive infection.

One important question is: what happens with the (majority of) HSV-1 genomes that do not lead to lytic infection? Are they in a latent state ? Although not all latently infected neurons express (detectable) latency-associated transcripts (LATs), it would be interesting to determine whether these non-lytically infected neurons express LATs, as this would clearly point in the direction of latency (Wagner and Bloom, 1997). In case LAT expression would be observed, localization of the LAT transcripts may point to latent versus lytic infection. Indeed, it has been described that the stable LAT 2kb intron can be exported to the cytoplasm during lytic infection as part of the 60S ribosomal subunit (Atanasiu and Fraser, 2007). Hence,

nuclear LAT would point to latent neurons. Another possibility to address whether non-lytically infected neurons may contain latent HSV-1, is the use of an HSV-1 LAT-specific *in situ* hybridisation assay (Theil et al., 2001; Gussow et al., 2006). Alternatively, earlier studies using an HSV-1 mutant that expresses β-galactosidase from the LAT promoter (LβA HSV-1) and our own studies (Chapter 3) comparing IFN-treated versus non-treated HSV-1-infected TG cultures, showed a distinct localization of LacZ in lytic and latent infected neurons. Lytic infected neurons showed a cytoplasmic distribution of the LacZ staining, while latently infected neurons had a focal staining (Ho and Mocarski, 1989; Lachmann and Efstathiou, 1997).

Interestingly, triggering reactivation in IFN-treated HSV-1-infected porcine TG neurons resulted in 11% of the accessible neurons that initiated expression of glycoprotein gD, a marker for productive infection. This percentage is very similar to the percentage of neurons that is able to proceed to productive infection during acute infection in the absence of IFN. This suggests that the cells that do not proceed to lytic infection during acute infection in the absence of IFN also do not respond to the reactivation stimulus (in our case forskolin). As described above, $A5^+$ neurons are non-permissive for lytic infection of HSV-1, but are also non-permissive for reactivation upon addition of the HDAC inhibitor trichostatin A (TSA) (Bertke et al., 2011). The $A5^+$ neurons that are non-permissive for lytic replication are mostly positive for TrkA, while other neurons that do support lytic replication are GDNF positive (Bertke et al., 2011). Interestingly, signalling through Trk receptor appears to be required to maintain latency of HSV-1, while this is not the case for signalling through GDNF (Camarena et al., 2010). Hence it might be interesting to study the presence of TrkA and/or GDNF in the *in vitro* system: in the subpopulation of neurons that shows expression of lytic viral genes during acute infection and reactivation, versus the (large) subpopulation that does not show such expression.

How does IFNα cause alphaherpesvirus latency-like quiescence in the TG cultures ? IFNα–induced latency in the two-chamber model correlated with the IFNα–induced suppression of expression of the immediate early viral protein ICP4 in HSV-1 and its counterpart in PRV, IE180. This ICP4/IE180 suppression was much more efficient in HSV-1 versus PRV, which correlated with the more efficient IFNα–mediated establishment of latency in HSV-1-infected versus PRV-infected TG neurons. As working with the primary TG neurons in the *in vitro* two-chamber posed too many technical difficulties, the question how IFN is causing latency-like quiescence was further addressed using a continuous cell line originating from rat dorsal root ganglion neurons (50B11s) (Chen et al., 2007). First, it was determined whether IFN caused similar effects on HSV-1/PRV replication in 50B11 cells compared to TG neurons. One difference was that the addition of IFNα alone was insufficient

to generate a very substantial reduction in HSV-1/PRV replication (data not shown). We found that the combination of IFNα and IFNγ resulted in the best suppression of viral replication, which is in line with other reports (Sainz and Halford, 2002). This combinatorial treatment was used in all following experiments.

Our data in general indicated that the 50B11 cells and the primary TG neurons share important similarities with regard to the effect of IFN on PRV/HSV-1 infection. However, one needs to keep in mind that important differences were also obvious between the 50B11 continuous cell line and the primary TG neurons. The first difference was the amount of IFN needed to suppress the infection. In TG neurons, infection was efficiently reduced using 500U/ml IFNα, while in 50B11 a combination of 1000U/ml IFNα and 100ng/ml IFNγ was needed to substantially suppress replication. Despite these higher amounts (and combination) of IFN, stable latency-like quiescence was never obtained in 50B11s, as in long term infections, CPE would eventually show up. Several hypotheses to explain these differences between TG neurons and 50B11 cells may be put forward. First, the 50B11 cell line was not cultured in a two-chamber system. Therefore, virus did not need to engage in retrograde transport to reach the cell nucleus, which likely will result in much higher levels of the viral transactivator VP16 reaching the nucleus. As the 50B11s do not establish a stable axon network, it is not possible to set up a two chamber system using 50B11 cells instead of TG neurons. In addition, it is likely that in 50B11 cells, which have the ability to replicate indefinitely, expression levels of cellular transcription factors such as HCF and Oct-1 and other cellular factors (e.g. cell cycle proteins) that affect HSV-1 replication may be substantially higher than in primary TG neurons. It is also possible that the fact that 50B11, which originate from rat, instigate more difficulties for the human IFNα to suppress the viral replication. Although this would not solve the issues related to cellular transcription/replication factors inherent to continuous cell lines compared to primary cells, it might be interesting to create a continuous cell line originating from porcine TGs to solve at least some of the issues mentioned above.

Notwithstanding the issues mentioned above comparing 50B11 and TG neurons, interesting similarities were observed that allowed to lift a tip of the veil regarding how IFN may aid in establishing alphaherpesvirus latency and why there is a difference in efficiency in IFN-mediated suppression of immediate early gene expression in HSV versus PRV. Like in the TG neurons, IFN treatment caused suppression of ICP4 and IE180 protein levels. Again, this IFN-mediated suppression was more efficient for HSV-1 ICP4 (from 4hpi onwards) compared to PRV IE180 (from 8hpi onwards). This was not due to differences in IFN-mediated suppression of mRNA levels of ICP4/IE180, as both were only suppressed

relatively late in infection (8hpi) and to comparable levels. Our data suggest that IFN efficiently and rapidly inhibits translation of HSV-1 mRNAs, while for PRV this is not the case.

Addition of IFN resulted in a suppression of mRNA levels of both HSV-1 ICP4 and PRV IE180, relatively late in infection (8hpi) of 50B11 cells. A transcriptional block of HSV-1 and PRV IE genes had been reported before, although the underlying mechanism remains unknown. Both *in vivo* and *in vitro* studies showed that IFN is able to block transcription at the immediate-early level (Gloger and Panet, 1984; Oberman and Panet, 1988; De Stasio and Taylor, 1990; Nicholl and Preston, 1996; Yao et al., 2007). To investigate the underlying mechanism for the transcriptional block, several paths need to be explored. First, there are several RNA degrading/modulating proteins that are upregulated by IFN that might be involved, e.g. APOBEC3, TRIMα, ADAR1 and RNaseL (Liu et al., 2010). RNA degradation is not likely to be the main cause of suppressed ICP4/IE180 mRNA levels, as cellular GAPDH mRNA levels were not affected by IFN or infection. RNA editing, e.g. through APOBEC3 or ADAR1 may affect successful translation of mRNA but likely would not interfere with RT-qPCR-based detection of these mRNAs, arguing against the possibility that RNA editing causes ICP4/IE180 mRNA suppression. Also, these RNA editing proteins have mainly been reported to affect replication of RNA viruses, including IFN-mediated suppression of HIV-1 replication (Argyris et al., 2007). Recently it was described that APOBEC3C is able to suppress viral replication of HSV-1, but through an IFN-independent pathway as APOBEC3C is not affected by IFN (Jarmuz et al., 2002; Suspene et al., 2011).

Alternatively or in addition to the above-mentioned effects of IFN on mRNA levels, it may be that IFN affects chromatin modifications that promote a closed chromatin structure. Histone deacetylases (HDACs) have been reported to promote such chromatin modifications, and, consequently, the inhibitor of HDACs trichostatin A (TSA) may activate the alphaherpesvirus genome (Poon et al., 2003). Our data show that HDAC is at least involved in the IFN-mediated suppression of transcription of HSV-1 ICP4, since TSA partially reverses the effect of IFN on ICP4 mRNA and protein levels. It might be possible that IFN primes the cell to immediately silence the incoming viral genome through the CoREST/REST/HDAC/LSD1 respressor complex, as we observed that CoREST is upregulated by IFN treatment. However, there is no universal agreement over the presence of REST (Repressor Element-1 Silencing Transcription) and CoREST (Corepressor of REST) in neurons as the main function of these proteins is the repression of neuronal genes in non-neuronal cells (Qureshi et al., 2010). However, an abundant amount of REST was detected in TGs of uninfected mice (Du et al., 2010) and CoREST staining on 50B11 cells showed a clear nuclear signal indicating that REST and CoREST might be present in neurons. It was shown before that the CoREST/REST/HDAC/LSD1 repressor complex plays a central role in

silencing the HSV-1 genome (Gu et al., 2005; Gu and Roizman, 2009b; Gu and Roizman, 2009a; Zhou et al., 2011). Recently, it was described that in mice, HSV-1 hijacks the CoREST/REST/HDAC/LSD1 repressor complex to silence itself as a mutant HSV-1 expressing dnREST is more virulent then WT virus (Du et al., 2010). It is possible that in the presence of IFN, the repressor complex is stimulated to silence the viral genome more efficiently than the virus would do itself. It might be interesting to investigate, e.g. through chromatin immunoprecipitation assays (ChIP), the chromatin structure on the viral genome in cells infected with or without IFN pre-treatment. Detection of acetyl groups on histones (especially H3) might give a clue towards the state of the chromatin as acetylation results in open chromatin structure while deacetylation causes a repressed chromatin structure. In addition, the genome may be assessed for increased repressive methylation markers (H3K9me2) and decreased activating methylation markers (H3K4me3) upon IFN treatment.

Although the effect of IFN on ICP4 and IE180 mRNA levels was similar, the effect on the protein level of these genes was quite different. The fact that, both in TG neurons and 50B11 cells, ICP4 was very quickly translationally repressed upon IFN treatment while IE180 was not, came as a surprise as HSV-1 encodes two viral proteins, ICP34.5 and US11, that counteract the IFN-mediated phosphorylation of the eukaryotic translation initiation factor eIF2α resulting in repression of translation. PRV does not encode orthologs for either of these proteins. Nevertheless, our data show that PRV is able to efficiently counteract the IFN-mediated phosphorylation of eIF2α in 50B11 cells, much more efficiently than HSV-1. It appears that the IFN-mediated suppression of HSV-1 replication in 50B11 cells is so efficient and rapid that ICP34.5 and US11, both expressed as (leaky-)late proteins, may not get the chance to function. As the dephosphorylation of eIF2α mediated by PRV occurs very early after infection (from 2hpi onwards), this points to the involvement of a tegument protein or an (immediate) early protein. Our data show that the PRV immediate-early protein IE180 is responsible and sufficient for the dephosphorylation of eIF2α. By encoding an immediate-early protein with the ability to counteract eIF2α phosphorylation, PRV resembles VZV, which encodes the immediate-early protein IE63 that causes dephosphorylation of eIF2α through a still unknown mechanism (Ambagala and Cohen, 2007). Although IE180 and IE63 are not orthologs, both closely related viruses use a similar manner of counteracting IFN-mediated repression of translation. All this suggests that these viruses may have gained their ability to counteract IFN-mediated phosphorylation of eIF2α relatively late in evolution, after the Simplexvirus genus has split from the Varicellovirus genus. Further research is required to determine whether IE180/ICP4 orthologs of HSV-1, BHV-1 and EHV-1 are also able to play a role in this process. The *in vivo* relevance of the immediate-early protein IE180 as a factor important for the dephosphorylation of eIF2α is not yet clear. ICP34.5$^{-/-}$ HSV-1 strains lost

their neurovirulence as the absence of ICP34.5 renders them extremely sensitive to IFNs (Leib et al., 1999). Similar experiments with PRV to assess the in vivo importance of IE180-mediated dephosphorylation of eIF2α will be less straightforward as IE180 mutants are unable to replicate, since IE180 is required for onset of transcription of the other viral genes. Hence, in order to be able to assess the in vivo relevance of our findings, it may be important to determine which domains in IE180 are involved in eIF2α dephosphorylation. If these domains are separatable from the parts of the protein involved in its transactivating function, mutants may be generated that still replicate but not longer dephosphorylate eIF2α.

Our data show the involvement of protein phosphatase 1 in dephosphorylation of eIF2α, which resembles the activity of ICP34.5 in HSV-1. Hence, although the viral protein may be completely different, the underlying mechanism of eIF2α dephosphorylation may be similar. Although future research is required to study whether IE180, like ICP34.5, is also able to interact directly with PP1, no sequence homologies were found to point in that direction. At first glance, it would appear more likely that IE180 induces the expression of a protein involved in PP1-mediated dephosphorylation of eIF2α (but not PP1 itself), which may then recruit PP1 to dephosphorylate eIF2α, such as GADD34 or CReP. Further research, using Western blot assays to determine expression levels of PP1-affecting proteins and immuno- precipitation assays to determine binding partners of IE180, will be needed to explore these different possibilities.

Conclusions and general hypotheses (Figure 1)

Based on the findings in the current thesis, we hypothesize that interferon promotes the establishment of alphaherpesvirus latency in sensory neurons, likely through inhibition of immediate-early gene expression both at the mRNA and protein level.

We hypothesize that the inhibitory effect of IFN on IE mRNA expression occurs partly through histone modifications, including histone deacetylases and the induction of components involved in repressor complexes like CoREST/REST/HDAC/LSD1.

As a countermeasure towards the suppressive effects of IFN on viral replication, we have found that the IE180 protein of PRV causes dephosphorylation of eIF2α, at least partly through the cellular phosphatase PP1. We hypothesize that IE180 causes these effects either through upregulation of components involved in PP1-mediated effects (apart from PP1 itself) or through establishing interactions with and thereby affecting the activity of PP1 or PP1-regulating factors.

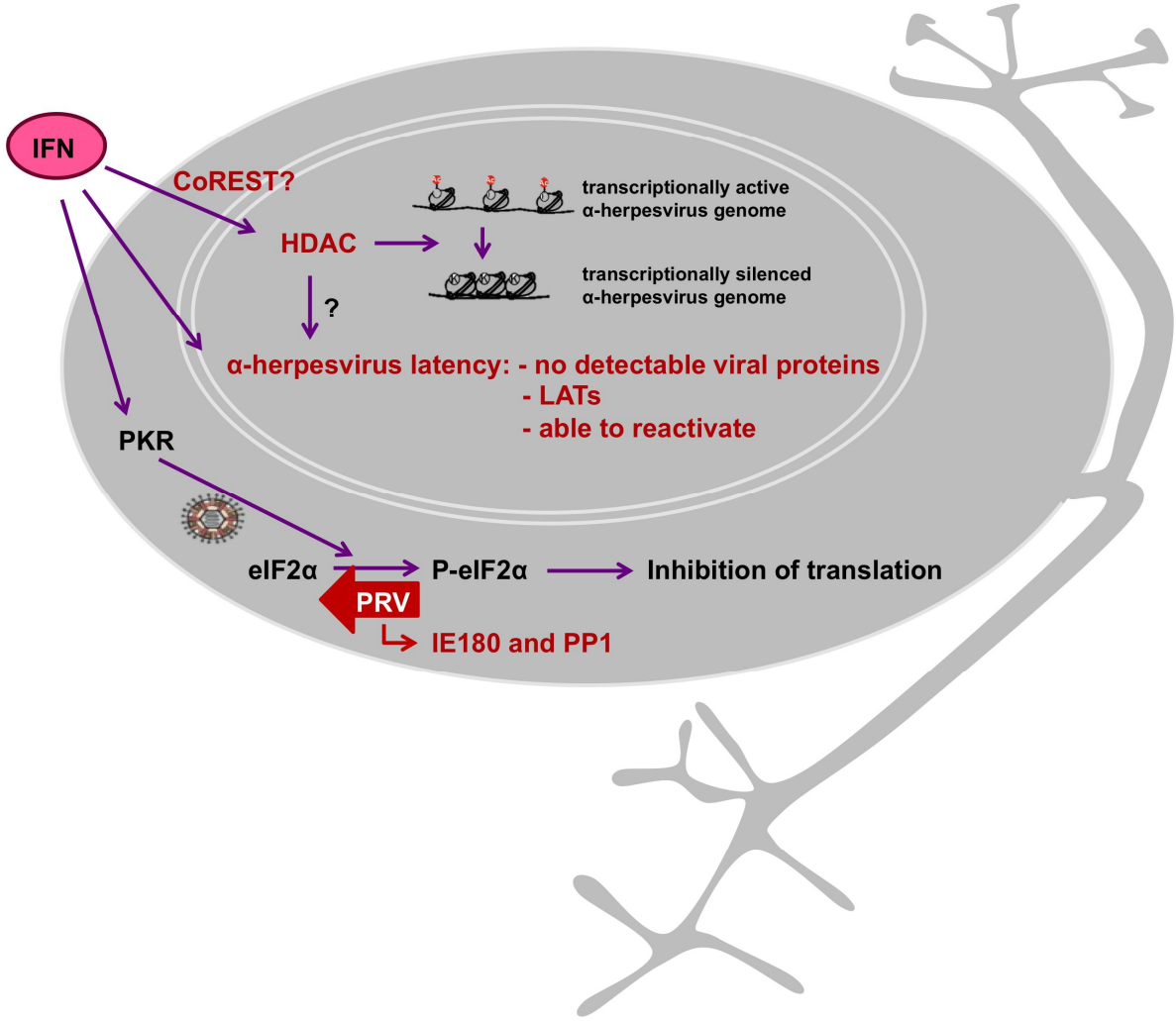


**Figure 1: Hypothetical model to explain the interactions of alphaherpesviruses, interferon, and sensory neurons observed in the current thesis.** We observed that IFN is able to induce HSV-1 and PRV latency in porcine TG neurons *in vitro*. We also found that IFN acts on alphaherpesvirus immediate early gene expression through the activity of HDACs (histone deacetylases) and that IFN upregulates protein levels of CoREST, an important component of the transcription suppressing CoREST/REST/HDAC/LSD1 complex. These effects potentially may all contribute to the ability of IFN to induce latency *in vitro*. In addition, we found that PRV efficiently counteracts phosphorylation of eIF2α, a translation-inhibiting consequence of IFN. We identified the immediate early IE180 protein of PRV as being able to suppress phosphorylation of eIF2α and demonstratedthe involvement of the cellular phosphatase PP1. Observations made in this thesis are indicated in red.

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## Chapter 8: Summary – Samenvatting

Herpesviruses share the common characteristic of being able to establish a latent infection in sensory neurons. It has become increasingly clear that the host immune system, the neuron and the virus act as a triple entente to establish and maintain viral latency. The major aim of this thesis was to gain a better insight in the interaction between the immune system, the neuron and the virus.

The first chapter consists of a brief introduction on alphaherpesviruses in general, on the sensory neurons where alphaherpesvirus latency is commonly established, and on alphaherpesvirus latency. Since interferon, a potent antiviral component of the immune respons, is of major importance in the current thesis, an overview of the antiviral effect of interferon and of alphaherpesvirus countermeasures is also given in this introductory chapter.

Chapter 2 describes the aims of this thesis.

In Chapter 3, using an *in vitro* model that enables a natural route of alphaherpesvirus infection, we showed that interferon alpha (IFNα) has the capacity to induce a quiescent HSV-1 and PRV infection in porcine TG neurons which shows strong similarity to *in vivo* latency. Latency of HSV-1 and PRV is characterized by the absence of viral proteins but the presence of viral DNA that can be reactivated upon specific stress stimuli. Addition of IFNα to the *in vitro* cultured primary TG neurons induced a stably suppressed HSV-1 and PRV infection in 100% and 45% respectively of the neurons growing axons into the outer chamber. HSV-1 and PRV latency *in vivo* is often accompanied by the expression of latency associated transcripts (LATs). RT-PCR was used to detect the latent transcript LAT or lytic transcripts IE180 and gB (PRV) or ICP4 and gD (HSV-1). Both for PRV and HSV-1, productively infected neurons were positive for both lytic and latent transcripts. Quiescent TG cultures were either only positive for LAT, or positive for both LAT and ICP0 (the latter suggestive for neurons on the verge of reactivation). Infection of TG neurons with an HSV-1 mutant expressing LacZ under control of the LAT promoter confirmed activation of the LAT promoter in quiescent TG cultures. For both viruses, treatment of quiescently infected neurons with forskolin resulted in reactivation. Together, these data represent a unique *in vitro* model of alphaherpesvirus latency and indicate that IFNα may be a driving force in promoting efficient latency establishment.

In addition, establishment of latency *in vitro* by the addition of IFNα correlated with suppressed expression of immediate-early (IE) genes ICP4 (HSV-1) and IE180 (PRV). For HSV-1, expression of ICP4 protein was suppressed fast en efficiently, correlating with efficient latency establishment *in vitro*. For PRV however, IE180 protein expression was less efficient and correlated well with the percentage of latency establishment *in vitro*. This

suggest IFNα-mediated suppression of IE expression like ICP4 and IE180 may be important in promoting alphaherpesvirus latency.

Since IFN-induced latency-like quiescence correlated with suppression of the immediate-early protein ICP4 in HSV-1 and its orthologue IE180 in PRV, in Chapter 4, we investigated the IFN-mediated suppression of ICP4 and IE180 in sensory neuronal cells. As working with primary TG neurons posed too many technical difficulties to address this, we opted to use a continuous cell line originating from rat dorsal root ganglion neurons, 50B11 cells. RT-qPCR assays showed that mRNA levels of either HSV-1 ICP4 or PRV IE180 at were only mildly but not significantly suppressed in IFN-treated samples at 4hpi, whereas a strong reduction was observed at 8hpi and 12hpi. However, when looking at protein levels using Western blot, at 4hpi, HSV ICP4 but not PRV IE180 protein expression was strongly reduced in IFN-treated samples. Hence, this indicates that for HSV-1, IFN results in rapid suppression of ICP4 protein levels, possibly through inhibition of translation, and a slower suppression of ICP4 mRNA levels, possibly through inhibition of transcription. PRV, on the other hand, appears to escape to some extent from IFN-mediated fast suppression of IE180 protein levels. To explain this difference in ability of IFN to suppress either HSV-1 ICP4 or PRV IE180 protein levels, the phosphorylation level of the translation initiation factor eIF2α was assessed. Indeed, one of the antiviral effector mechanisms of interferon consists of PKR-mediated phosphorylation of translation initiation factor eIF2α, resulting in shutdown of translation. In line with the difference in IFN-mediated suppression of HSV ICP4 versus PRV IE180 protein levels, we found that IFN resulted in an increase in phosphorylation of the translation initiation factor eIF2α in HSV-infected but not in PRV-infected cells. The latter finding indicates that PRV efficiently circumvents IFN-mediated translation inhibition by interfering with phosphorylation of eIF2α.

As Chapter 4 showed that IFN pre-treatment resulted in a decrease of HSV-1 ICP4 (and PRV IE180) mRNA levels later in infection, we set out to study this apparent block in transcription in Chapter 5. Over the past decade, it has already been suggested extensively that transcription of alphaherpesviruses is controlled by chromatin modifications. Specifically, the repressive CoREST/REST/HDAC/LSD1 complex controls transcription of the viral genome and has been suggested to be involved in control of alphaherpesvirus latency. Hence, we investigated whether histone modifications may be involved in IFN-mediated suppression of ICP4 in HSV-1 infected cells. First, the involvement of histone deacetylases (HDACs) was evaluated using the histone deacetylase inhibitor trichostatin A (TSA). RT-qPCR analysis showed that treatment with TSA partially counteracted the suppressive effect of IFN on ICP4 mRNA levels. Subsequently immunofluorescent staining and Western blotting for ICP4 confirmed these findings. These data suggest that the IFN-mediated suppressive

effect on ICP4 mRNA and protein expression includes the involvement of histone deacetylases. In a next step, protein levels of several components of the CoREST/REST/HDAC/LSD1 repressor complex were compared in mock versus IFN-treated samples. IFN was found to induce CoREST expression, while LSD1 and HDAC expression levels remain unchanged. Based on these data, we have indications that the suppressive effects of IFN on ICP4 levels may involve gene silencing factors, including histone deacetylases.

Since Chapter 4 showed that PRV appears to efficiently circumvent IFN-mediated translation inhibition by interfering with phosphorylation of eIF2α in 50B11 cells, we investigated the mechanism of PRV-mediated suppression of eIF2α phosphorylation in Chapter 6. Inhibition of phosphorylation of eIF2α has been reported earlier for HSV-1 through its ICP34.5 protein. PRV however does not encode an orthologue of ICP34.5. We report that PRV actively dephosphorylates eIF2α, rather than preventing its phosphorylation as PRV is not only able to remove basal levels of eIF2α phosphorylation, but also thapsigargin-pre-induced levels. Assays using cycloheximide (inhibitor of protein synthesis) or phosphonoacetic acid (inhibitor of replication and therefore alphaherpesvirus late protein expression) showed that *de novo* expression of one or more (immediate) early viral protein(s) was required for eIF2α dephosphorylation. Infection with UV-inactivated PRV confirmed these results. PRV encodes only one immediate-early protein, IE180, the orthologue of HSV-1 ICP4. A combinational treatment of 50B11 cells with cycloheximide and actinomycin D allowed the sole expression of IE180 in PRV infected cells which still resulted in a substantial reduction in eIF2α phosphorylation, indicative for an involvement of IE180 in this process. In support of this, transfection of IE180 also reduced phosphorylation of eIF2α. Taken together, we show that IE180 causes dephosphorylation of eIF2α. These data were confirmed in porcine ST cells. Active dephosphorylation of eIF2α implies the involvement of a cellular phosphatase as the virus does not encode a viral phosphatase. By using a PP1-specific inhibitor we showed that inhibition of the cellular phosphatase PP1 interfered to some extent with PRV-mediated dephosphorylation of eIF2α, indicating that PP1 may be involved in the IE180-mediated dephosphorylation of eIF2α, although IE180 did not upregulate PP1 protein expression. In conclusion, the immediate-early IE180 protein of PRV has the previously uncharacterized ability to cause dephosphorylation of the eukaryotic translation initiation factor eIF2α.

In conclusion, based on the findings in the current thesis, we hypothesize that interferon promotes the establishment of alphaherpesvirus latency in sensory neurons, likely through inhibition of immediate-early gene expression both at the mRNA and protein level.

We hypothesize that the inhibitory effect of IFN on IE mRNA expression occurs partly through histone modifications, including histone deacetylases and the induction of components involved in repressor complexes like CoREST/REST/HDAC/LSD1. As a countermeasure towards the suppressive effects of IFN on viral replication, we have found that the IE180 protein of PRV causes dephosphorylation of eIF2α, at least partly through the cellular phosphatase PP1. We hypothesize that IE180 causes these effects either through upregulation of components involved in PP1-mediated effects (apart from PP1 itself) or through establishing interactions with and thereby affecting the activity of PP1 or PP1-regulating factors.

**Samenvatting**

Herpesvirussen hebben de unieke eigenschap dat ze een latente, slapende infectie kunnen veroorzaken. Alfaherpesvirussen veroorzaken dergelijke latente infectie veelal in sensorische neuronen. Het wordt steeds duidelijker dat het gastheer immuunsysteem, het neuron en het virus zich gedragen als een “triple entente” om een latente infectie in te stellen en te onderhouden. De belangrijkste doelstelling van deze thesis was om een beter inzicht te verwerven in de interactie tussen het immuunsysteem, het neuron en het virus.

Het eerste hoofdstuk omvat een korte inleiding over alfaherpesvirussen in het algemeen, over de neuronen waarin latentie optreedt door alfaherpesvirussen en over alfaherpesvirus latentie zelf. Aangezien interferon, een sterke antivirale component van het immuunsysteem, een belangrijk element vormt in deze thesis, wordt in dit inleidende hoofdstuk ook een overzicht gegeven van de antivirale effecten van interferon en de mechanismen die alfaherpesvirussen hebben ontwikkeld om deze antivirale effecten te omzeilen.

Hoofdstuk 2 beschrijft de doelstellingen van deze thesis.

In hoofdstuk 3, hebben we, gebruik makend van een *in vitro* model dat het mogelijk maakt om de natuurlijke infectie route van een alfaherpesvirus infectie in sensorische neuronen na te bootsen, aangetoond dat interferon alfa (IFNα) in staat is om een slapende HSV-1 en PRV infectie te veroorzaken die sterke gelijkenissen vertoont met *in vivo* latentie. Latentie bij HSV-1 en PRV wordt gekarakteriseerd door de afwezigheid van virale eiwitten, maar de aanwezigheid van viraal DNA dat kan reactiveren onder invloed van bepaalde stress factoren. Toediening van IFNα aan de *in vitro* gecultiveerde primaire TG neuronen resulteerde in een stabiel onderdrukte HSV-1 en PRV infectie in respectievelijke 100% en 45% van de voor het virus toegankelijke. HSV-1 en PRV latentie *in vivo* wordt vaak vergezeld door de expressie van latentie-geassocieerde transcripten (LATs). RT-PCR werd gebruikt voor de detectie van LAT of de lytische transcripten IE180 en gB (PRV) of ICP0 en gD (HSV-1). Zowel voor PRV als HSV-1 waren productief geïnfecteerde neuronen positief voor zowel lytische als latente transcripten. De TG culturen die enkel door IFNα onderdrukt virus bevatten waren ofwel enkel positief voor LAT, ofwel positief voor zowel LAT als ICP0 (dit suggereert dat deze neuronen op de overgang van latentie naar reactivatie staan). Infectie van TG neuronen met een HSV-1 mutant die LacZ tot expressie brengt onder controle van de LAT promoter bevestigde de activatie van de LAT promoter in TG culturen met onderdrukte virusinfectie. Voor beide virussen resulteerde behandeling van deze culturen met forskoline in reactivatie. Op basis van deze gecombineerde data kan gesteld worden dat een uniek *in vitro* model voor alfaherpesvirus latentie werd opgesteld. De data

impliceren daarenboven dat IFNα een drijvende kracht kan vormen bij het instellen van alfaherpesvirus latentie in sensorische neuronen. Interessant hierbij was dat het instellen van latentie *in vitro* door toediening van IFNα correleerde met de onderdrukking van de expressie van immediate early (IE) genen ICP4 (HSV-1) en IE180 (PRV). Bij HSV-1 werd de expressie van ICP4 snel en efficiënt onderdrukt door IFNα, wat correleerde met de efficiënte instelling van latentie van dit virus *in vitro*. Voor PRV, daarentegen, werd het IE180 eiwit minder efficiënt onderdrukt en in een lager percentage cellen wat ook correleerde met het percentage van latentie *in vitro*. Dit suggereert dat de IFN-gemedieerde onderdrukking van IE expressie zoals ICP4 en IE180 belangrijk kan zijn bij de overgang naar een latente alfaherpesvirus infectie.

Aangezien IFN-geïnduceerde latentie *in vitro* bleek te correleren met de IFN-gemedieerde onderdrukking van de IE eiwitten ICP4 van HSV-1 en zijn ortholoog IE180 van PRV, werd in hoofdstuk 4 onderzocht op welk niveau (mRNA en/of eiwit) de IFN-gemedieerde onderdrukking van ICP4 en IE180 in sensorische neuronen plaatsvindt. Aangezien het werken met primaire TG neuronen teveel technische problemen met zich meebracht om dit te ontrafelen, werd geopteerd voor het gebruik van een continue cellijn afkomstig van rat DRG neuronen, 50B11 cellen. Door gebruik te maken van RT-qPCR werden de mRNA expressieniveaus vergeleken van ICP4 (HSV-1) en IE180 (PRV) in stalen zonder en met IFN voorbehandeling. Voor beide virussen waren de resultaten gelijkaardig. Het bleek dat expressie van zowel ICP4 als IE180 op 4hpi niet significant verschillend was met of zonder IFN behandeling terwijl een sterke reductie werd waargenomen in de IFN-behandelde stalen op 8hpi en 12hpi. Echter, bij het bestuderen van de eiwitniveaus bleek dat op 4hpi de ICP4, maar niet de IE180 expressie, sterk gereduceerd was in IFN behandelde stalen. Dit impliceert dat voor HSV-1, IFN behandeling resulteert in een snelle onderdrukking van ICP4 eiwitniveaus mogelijk door reductie van translatie. Voor beide virussen is er een (tragere) onderdrukking van ICP4/IE180 mRNA gehaltes, mogelijk door inhibitie van transcriptie. PRV lijkt echter te ontsnappen aan de snelle IFN-gemedieerde onderdrukking van IE180 eiwitgehaltes. Om het verschil te verklaren in capaciteit van IFN om enerzijds HSV-1 ICP4 en anderzijds PRV IE180 eiwitgehaltes te onderdrukken, werd het niveau van fosforylatie van de eukaryote translatie initiatie factor eIF2α bepaald. Eén van de sterkste antivirale effector mechanismen van IFN bestaat immers uit PKR-gemedieerde fosforylatie van de translatie initiatie factor eIF2α wat resulteert in het inhiberen van translatie. In overeenstemming met het waargenomen verschil in IFN-gemedieerde onderdrukking van ICP4 versus IE180 proteïneniveaus, bleek dat IFN behandeling zorgde voor een verhoogde fosforylatie van eIF2α in HSV-1-, maar niet in PRV-geïnfecteerde cellen. Dit wijst erop dat

PRV in staat is om op een efficiënte manier de IFN-gemedieerde inhibitie van translatie tegen te werken door te interfereren met de fosforylatie van eIF2α.

Aangezien hoofdstuk 4 toonde dat IFN voorbehandeling resulteerde in een onderdrukking van HSV-1 ICP4 (en PRV IE180) mRNA niveaus tijdens latere stadia van infectie, werd deze ogenschijnlijke blokkade van transcriptie verder bestudeerd in hoofdstuk 5. Steeds meer data suggereren dat transcriptie van alfaherpesvirussen gecontroleerd wordt door chromatine veranderingen. Vooral het repressieve CoREST/REST/HDAC/LSD1 complex blijkt transcriptie van het virale genoom te controleren en werd gesuggereerd betrokken te zijn bij alfaherpesvirus latentie. Daarom werd de rol van histon-modificaties bestudeerd in de IFN-gemedieerde onderdrukking van ICP4 in HSV-1 geïnfecteerde cellen. Eerst werd de betrokkenheid van histon deacetylasen (HDACs) geëvalueerd door gebruik te maken van de inhibitor trichostatin A (TSA). RT-qPCR analyse toonde dat behandeling van TSA het onderdrukkend effect van IFN op ICP4 mRNA niveaus gedeeltelijk kon tegenwerken. Vervolgens werd dit bevestigd op eiwitniveau door zowel immunofluorescente kleuringen als Western blotting. Deze data suggereren dat het IFN-gemedieerde onderdrukkende effect op ICP4 expressie deels veroorzaakt wordt door betrokkenheid van histon deacetylases. In een volgende stap werden de eiwitniveaus van verschillende componenten van het CoREST/REST/HDAC/LSD1 repressorcomplex vergeleken in mock versus IFN-behandelde stalen. IFN bleek in staat om CoREST expressie te verhogen, terwijl LSD1 en HDAC expressie onveranderd bleef. Deze data suggereren dat het onderdrukkende effect van IFN op ICP4 mede het gevolg kan zijn van genoom silencing waarbij histon deacetylasen betrokken zijn.

Aangezien in hoofdstuk 4 aangetoond werd dat PRV in staat is om op een efficiënte manier de IFN-gemedieerde inhibitie van translatie tegen te werken door te interfereren met de fosforylatie van eIF2α in 50B11 cellen, werd in hoofdstuk 6 het mechanisme hiervan verder onderzocht. Inhibitie van fosforylatie van eIF2α werd eerder beschreven voor HSV-1 via het ICP34.5 eiwit van dit virus. PRV echter heeft geen ortholoog voor ICP34.5. Verlaagde fosforylatieniveaus van eIF2α kunnen te wijten zijn aan verhinderde fosforylatie van dit eiwit of actieve defosforylatie van het eiwit. PRV bleek te zorgen voor actieve defosforylatie eerder dan voorkomen van fosforylatie aangezien zowel basale als thapsigargine-geïnduceerde niveaus van fosforylatie van eIF2α teniet gedaan werden door PRV infectie. Om na te gaan welk viraal eiwit verantwoordelijk is voor deze defosforylatie van eIF2α, werd eerst het stadium van infectie bepaald waar defosforylatie optreedt. Experimenten met cylcoheximide (inhibitor van proteine synthese) en phosphonoacetic acid (inhibitor van replicatie en daardoor expressie van late eiwitten van alfaherpesvirussen) toonden aan dat *de novo* expressie van één of meer (I)E virale eiwitten nodig zijn om eIF2α te defosforyleren. Infectie

experimenten met UV geïnactiveerd PRV bevestigde deze resultaten. PRV codeert slecht voor één IE eiwit, namelijk IE180, het ortholoog van ICP4. Een combinatie behandeling van cycloheximide en actinomycine D laat toe om enkel IE180 tot expressie te laten komen in PRV geïnfecteerde cellen. Deze behandeling resulteerde nog steeds in een substantiële reductie van gefosforyleerd eIF2α wat impliceert dat IE180 betrokken is bij dit proces. Om dit te bevestigen werden IE180 transfectie experimenten uitgevoerd, waarbij opnieuw een significante reductie van gefosforyleerd eIF2α werd bekomen. Deze data tonen dus aan dat IE180 verantwoordelijk is voor de defosforylatie van eIF2α. Deze data werden verder bevestigd in porciene ST cellen. Actieve defosforylatie van eIF2α impliceert de betrokkenheid van een cellulair fosfatase aangezien het virus zelf niet codeert voor een fosfatase. Door gebruik te maken van een inhibitor van het cellulaire fosfatase PP1 werd aangetoond dat PP1 betrokken lijkt te zijn bij de IE180-gemedieerde defosforylatie van eIF2α. IE180 zorgde echter niet voor een opregulatie van PP1 eiwitexpressie. Samenvattend werd aangetoond dat het IE eiwit IE180 van PRV de voorheen onbeschreven eigenschap heeft om defosforylatie van eIF2α te veroorzaken.

Gebaseerd op de bevindingen van deze thesis, stellen we de hypothese op dat interferon het instellen van een alfaherpesvirus latente infectie promoot in sensorische neuronen, vermoedelijk via inhibitie van IE gen expressie zowel op mRNA als eiwitniveau. We suggereren dat het inhibitorisch effect van IFN op IE mRNA expressie deels voorkomt uit IFN-geïnduceerde histon-modificaties, waarbij histon deacetylasen betrokken zijn, mogelijk via het repressorcomplex CoREST/REST/HDAC/LSD1. Om het onderdrukkende effect van IFN op virale vermeerdering te omzeilen blijkt IE180 van PRV aanleiding te geven tot defosforylatie van eIF2α, waarbij het cellulaire fosfatase PP1 deels betrokken lijkt te zijn. We suggereren dat IE180 dit effect veroorzaakt ofwel door opregulatie van componenten die betrokken zijn bij PP1-gemedieerde effecten (maar niet PP1 zelf) ofwel door een interactie aan te gaan met PP1 of PP1-regulerende factoren.

# Curriculum Vitae

Personalia

Nina Van Opdenbosch werd geboren op 19 oktober 1984 te Jette. Zij beëindigde haar secundaire studies in 2002 aan het Koninklijk Atheneum te Ninove (richting Wetenschappen-Wiskunde). Vervolgens startte zijn haar universitaire studies aan de Faculteit Bio-ingenieurswetenschappen van de Universiteit Gent waar zij in 2007 het diploma behaalde van Master in de Bio-ingenieurswetenschappen: cel- en genbiotechnologie met onderscheiding. Sinds augustus 2007 verricht zij onderzoek aan de Vakgroep Virologie, Parasitologie en Immunologie op de Faculteit Diergeneeskunde van de Universiteit Gent. Vanaf januari 2008 beschikt zij over een vierjarig IWT-doctoraatsbursaal van het Vlaamse Agentschap voor Innovatie door Wetenschap en Techniek. Dit onderzoek handelt over de interactie tussen interferon (een antivirale component van het immuunsysteem) zenuwcellen en alfaherpesvirussen tijdens het instellen van een latente alfaherpesvirus infectie in deze cellen.

Publicaties

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*Equally contributed

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